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委員会報告

う蝕予防のための日本人におけるフッ化物摂取基準（案）の作成

眞木 吉信[1,2]，荒川 浩久[1]，磯崎 篤則[1]，小林 清吾[1,2]
飯島 洋一[1,2]，田浦 勝彦[1]，古賀 寛[2]，西牟田 守[2]

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日本人におけるフッ化物摂取基準案作成にいたる経緯

日本歯科医学会医療問題検討委員会フッ化物検討部会は日本歯科医学会 斎藤 毅会長の要請を受け，平成10年1月22日，第一回の委員会を開催した．以来，平成11年10月8日までに9回の会議を開催し，「フッ化物応用についての総合的な見解」をまとめるために検討を重ねてきた．その結果，平成11年11月1日に答申を行い，国民の口腔保健向上のために，う蝕予防を目的としたフッ化物の応用を推奨するとともに，わが国におけるフッ素の適正摂取量を確定するための研究の推進を奨励することとなった．この答申に基づき，平成12年4月に厚生省（現厚生労働省）は「歯科疾患の予防技術・治療評価に関するフッ化物応用の総合的研究」(略称：フッ化物応用の総合的研究，H 12-医療-003，主任研究者 高江洲義矩，東京歯科大学教授)を発足させることとなった．

厚生労働科学研究「フッ化物応用の総合的研究」班によるフッ化物摂取基準の検討

厚生科学研究「歯科疾患の予防技術・治療評価に関するフッ化物応用の総合的研究」班（主任研究者 高江洲義矩）は，日本歯科医学会の「フッ化物応用についての総合的な見解」(平成11年）の報告を受けて発足し，3年後に，厚生労働科学研究「フッ化物応用による歯科疾患の予防技術評価に関する総合的研究」班（主任研究者 高江洲義矩，眞木吉信)に引き継がれた．さらに，3年間の研究として，「フッ化物応用による歯科疾患予防プログラムの構築と社会経済的評価に関する総合的研究」班(主任研究者 眞木吉信）において継続されている．

日本歯科医学会の「フッ化物応用についての総合的な見解」において，フッ化物応用の基礎となるフッ化物摂取基準を確定するための研究を推進することが提唱され，研究班における「フッ化物の栄養所要量と健康」グループに課せられたテーマは，わが国において健康増進のためのフッ化物応用を推奨していくために，乳児から成人，老人にいたる生涯を通したう蝕予防のためのフッ化物摂取の目安量（Adequate Intake；AI）および上限量(Tolerable Upper Limited Intake Level；UL)を策定することにあった．

フッ化物の栄養学的評価は，近年の微量元素の摂取基準がアメリカから発信された栄養摂取概念をもとに展開されており，日本においても第6次栄養摂取基準改定から援用されている．初期の厚生労働科学研究においては，各年齢群別におけるフッ化物摂取量に関する知見を収集するとともに，乳児，幼児および児童のフッ化物摂取に関する調査と実験研究を行った．また，母乳および日本において市販されている主な調製粉乳のフッ化物濃度の分析を通して乳児のフッ化物摂取量を推定した．その後，幼児（3～5歳児）のフッ化物摂取量を陰膳食法による食事調査から求め，さらに浄水場の平均フッ化物濃度も考慮して0.16 ppmF未満の低フッ化物濃度飲料水地区でのフッ化物摂取量を実際に求めた．その結果，平均値0.28 mg/day(1～6歳児)，および0.29 mg/day(3～5歳児)，フッ化物配合歯磨剤を含めた総フッ化物摂取量でも0.35 mg/day，最大値でアメリカの上限値(UL)を超えることなく，目安量（AI）の2分の1程度であった．

また，わが国におけるフロリデーション（水道水フッ化物添加）を考慮した幼児（3～5歳）のフッ化物摂取量を試算すると，食事からのフッ化物摂取量がアメリカの

[1]日本口腔衛生学会フッ化物応用委員会
[2]厚生労働科学研究「フッ化物応用による歯科疾患予防プログラムの構築と社会経済的評価に関する総合的研究」(H 18-医療-一般-019)

設定の目安量（AI）を満たし，上限量（UL）を超えない摂取量となり，0.8 ppmF の飲料水において平均値 0.73 mg と推定され，最大値でも上限量（UL：1.7 mg/day）を超えることなく，3 歳児の目安量（AI）程度と評価された．

次に，飲料水フッ化物濃度が異なる 2 つの地域の小児における食事からのフッ化物摂取量を陰膳食法で検討したところ，飲料水フッ化物濃度 0.6 ppm 地域の中学生は低濃度（0.1 ppmF 以下）地域の生徒と比べう蝕経験歯数が有意に少なく，歯のフッ素症も審美的に問題となるレベルの発現はないことが示された．

食品中フッ化物分析については，まず，普遍的なフッ化物分析法である微量拡散—フッ化物イオン電極による食品中フッ化物分析法の信頼性と妥当性を検証した．この方法では，無機のフッ化物添加回収実験で 91% 以上の回収率が得られ，数種類の食品を複数の研究機関で比較しても有意な差は認められないので，本法は食品のフッ化物分析法として適切であることが示された．

食品中フッ化物分析値においては，海産物を中心として，魚類 32 品目（可食部）のフッ化物濃度は，0.02～9.07 μg/g，変動係数 0.7～39.4% の範囲であった．そのなかでフッ化物濃度 1.0 μg/g 以上のものが，9 品目あった．さらに，マーケットバスケット方式によって国民栄養調査成績表（平成 11 年度）の分類に準じた，66 品目を分析したフッ化物濃度では，米 0.14 μg/g，小麦粉 0.03 μg/g と低値を示した．麺類 0.14 μg/g，砂糖 0.07 μg/g，乳製品 0.05 μg/g，魚介類（魚の可食部）0.44 μg/g，で魚介類が最も高い値を示した．肉や豆腐，野菜，果物，ジャガイモはおおむね 0.1 μg/g 以下の低値を示した．

これまでの日本における飲食物からのフッ化物摂取量の文献をレビューしたところ，飲食物からの 1 日あたりの総フッ化物摂取量は，成人では 0.89～5.4 mg/day と文献間のバラツキが大きいが，1990 年以降の報告では，0.90～1.28 mg/day であった．また，乳児ではドライミルクと乳児用食品を摂取した場合 0.09～0.27 mg/day，幼児では 0.23～0.38 mg/day であった．乳幼児における総摂取量はアメリカの設定基準（Dietary Reference Intakes；DRI）が示した AI（目安量）の約 2 分の 1 であった．

以上の研究知見をまとめた結果が表 1，2 に示した「日本人におけるフッ化物摂取基準（案）」である．

日本口腔衛生学会における「日本人におけるフッ化物摂取基準案」の検討と承認支援

日本口腔衛生学会では平成 19 年 3 月 1 日，厚生労働科学研究「フッ化物応用による歯科疾患予防プログラムの構築と社会経済的評価に関する総合的研究」（H 18-医療-一般-019），主任研究者　眞木吉信，東京歯科大学教授より，上記案の承認支援の依頼を受けて，フッ化物応用委員会において検討を重ね，その結果を全理事へ諮ったところ，この提案を支援するにいたった．

日本歯科医学会における「日本人におけるフッ化物摂取基準案」の推奨

アメリカやカナダに代表される北米やヨーロッパの先進諸国では，フッ化物が健康の保持，増進のための栄養素として認められ，摂取基準量が策定されている．一方，日本においても，前述したように日本歯科医学会の見解に基づき，フッ化物の摂取基準に関する研究が平成 12 年から厚生労働科学研究として実施されてきた．その研究成果として表 1，2 の「日本におけるフッ化物摂取基準（案）」が作成された．この基準案は日本口腔衛生学会においても承認と支援を受けて学術的にも問題のない数字が提示されたと考えられる．日本歯科医学会としても，健康の推進と疾病のリスク低減の観点で，この摂取基準（案）を推奨する立場から，厚生労働省の策定する「2010 年版日本人の食事摂取基準」に上記のフッ化物の摂取基準（案）の収載を依頼している．

日本人におけるフッ化物摂取基準（案）

生涯にわたる健康を維持・増進するうえで，フッ化物応用によるう蝕予防は基本的かつ不可欠であり，多くの疫学調査から実証されている[1,2]．このようなフッ化物の摂取基準は，アメリカでは推定平均必要量（EAR：estimated average requirement）の推定が困難なことから，各年齢層別の一日あたりのフッ化物の目安量（AI：adequate intake）と上限量（UL：tolerable upper intake level）が提示されている[3]．しかしながら，日本人の食事摂取基準では 2005 年版（2005 年～2009 年使用）現在においてもフッ化物の摂取基準は，いまだ設定されるにいたっていない[4]．フッ化物はあらゆる食品に含有されているため，その摂取基準の設定が困難であり，日本ではその基礎資料も示されていなかった．日本人の基準値を策定するには，フッ化物摂取のう蝕予防効果と過剰摂取

表1　ライフステージにおけるフッ化物摂取基準

年齢	フッ化物（mgF/日）					
	男			女		
	目安量（mg）	上限量（mg）	基準体重（kg）	目安量（mg）	上限量（mg）	基準体重（kg）
0–5（月）	母乳栄養児 0.01	0.66	6.6	母乳栄養児 0.01	0.61	6.1
0–5（月）	人工栄養児 0.33	0.66	6.6	人工栄養児 0.31	0.61	6.1
6–11（月）	0.44	0.88	8.8	0.41	0.82	8.2
1–2（歳）	0.60	1.19	11.9	0.55	1.10	11.0
3–5（歳）	0.84	1.67	16.7	0.80	1.60	16.0
6–7（歳）	1.15	2.30	23.0	1.08	2.16	21.6
8–9（歳）	1.40	2.80	28.0	1.36	2.72	27.2
10–11（歳）	1.78	6.0	35.5	1.79	6.0	35.7
12–14（歳）	2.50	6.0	50.0	2.28	6.0	45.6
15–17（歳）	2.92	6.0	58.3	2.50	6.0	50.0
18–29（歳）	3.18	6.0	63.5	2.50	6.0	50.0
30 歳以上	3.40	6.0	68.0	2.64	6.0	52.7

注 1）年齢層の区分は日本人の食事摂取基準（2005 年版）に依拠している．
注 2）母乳栄養児は母乳中フッ化物濃度が 0.01ppm（中央値）であり，摂取量 1000m*l* として算出した．

表2　妊婦・授乳婦のフッ化物摂取基準（mgF/日）

妊婦/授乳婦	目安量（mg）	上限量（mg）
妊婦	2.5	6.0
授乳婦	2.5	6.0

による危険性，すなわち，日本の小児における歯の審美的副作用（adverse cosmetic effect）である「歯のフッ素症（enamel fluorosis）」の発現とその基準値設定の基礎資料が必要となる．また，食品に嗜好飲料水や居住地域の水道水を含めた食事からのフッ化物摂取量と歯磨剤からの飲み込み量を合わせた総フッ化物摂取量の把握が必要である[5-7]．

2000 年 4 月に発足した厚生科学研究（現厚生労働科学研究）は「歯科疾患の予防技術・治療評価に関するフッ化物応用の総合的研究」（主任　高江洲義矩）から始まり，2003 年度には「フッ化物応用による歯科疾患の予防技術評価に関する総合的研究」，2006 年度には「フッ化物応用による歯科疾患予防プログラムの構築と社会経済的評価に関する総合的研究」（H 18-医療—一般-019）（主任　眞木吉信）に改組され，口腔保健に関するフッ化物応用の総合的研究を実施している．フッ化物摂取基準の策定は歯科保健を推進するうえで必須であり，ライフステージごとに飲食物からのフッ化物摂取量と歯磨剤の口腔内残留量も加味して，目安量（AI）と摂取上限量（UL）を設定した．

フッ化物摂取の目安量の基準は，疫学的調査からう蝕罹患率を有意に減少させる体重 1 kg あたり 0.02 から 0.05 mg/kg である事実[6,8-12]に基づいて，その高い値である 0.05 mg/kg とした．また上限量（UL）の基準は，LOAEL 値を参照した[3]．すなわち，MO（Dean の分類の modelate）の発現頻度が飲料水中フッ化物濃度 2 ppm 未満の場合では 5% 未満であるという疫学的事実[13]に基づいている．上限量の明確な計算過程は文献には示されていないが，推考すると次のような計算過程で求められていると考えられる．

1）飲料水中フッ化物濃度の最大値を 2 ppm とし，一日飲水量を 1.5 *l* とする．

①飲料水からのフッ化物量：2 mg/*l*×1.5 *l*＝3 mg/day

②食事からのフッ化物摂取量：0.25–0.3 mg/day

③フッ化物飲料水で調理した食事中フッ化物摂取量：0.3×2＝0.6 mg/day

①＋③最大一日フッ化物摂取量＝3＋0.6＝3.6 mg/day

2）飲料水中フッ化物濃度の最大値を 2 ppm とし，一日飲水量を 1.0 *l* とする．

①飲料水からのフッ化物量：2 mg/*l*×1.0 *l*＝2 mg/day

②食事からのフッ化物摂取量：0.25–0.3 mg/day

③フッ化物飲料水で調理した食事中フッ化物摂取量：0.25×2＝0.5 mg/day

①＋③最小一日フッ化物摂取量＝0.5＋2.0＝2.5 mg/day

8～9歳児の体重を約30 kg[4]と仮定すると，2)より，最小2.5/30＝0.083 mg/kg/day，1)より，最大3.6/30＝0.12 mg/kg/dayと計算される．すなわち，上限量の範囲は，0.083–0.12 mg/kg/dayとなる．そして，その平均値をとると0.1 mg/kg/dayとなる．どうして8～9歳児を基準としたかは，永久歯の発生学的解釈から成熟期と密接に関連[13]しているからである．したがって，上限量は0.1 mg/kg/dayと設定した．この上限量はフッ化物摂取による健康障害の発現ではなく歯の審美的副作用である[3]．この体重あたりの目安量と上限量に各年齢層の日本人の基準体重[4]を乗じて男女別に8～9歳までの摂取基準値を設定した（表1）．さらに「歯のフッ素症」のmoderateが進行する臨界副作用（critical adverse effect）の感受性年齢（susceptible age groups）は病理学的には8歳までである[14]．したがって，日本人の食事摂取基準の年齢区分における10歳以上の上限量は，成人の体重を約60 kg[4]と仮定して，0.1 mg/kg×60 kg＝6 mg/dayと推定し，男女ともに6 mg/dayに統一した（表1）．

また，妊婦と授乳婦における目安量と上限量の範囲では，母乳にはフッ化物は移行しない事実[15,16]，胎児への移行も制限されるという事実[17,18]から15～29歳の目安量と上限量と同じ値に設定した(表2)．表1，2の目安量と上限量は，食品，飲料水，栄養補助食品およびフッ化物配合歯磨剤からの摂取量である．

文　献

1) McDonagh M, Whiting, P, Bradly M et al.: A systematic review of public water fluoridation. The University of York, York, 2000.
2) U. S. Department of Health and Human Services: Recommendations for using fluoride to prevent and control dental caries in the United State. MMWR (Morbidity and Mortality Weekly Report) Vol. 50, No. RR-14, Centers for Disease Control and Prevention, Atlanta, 2001.
3) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, Food and Nutrition Board, Institute of Medicine: Dietary reference intakes for calcium, phosphorus, magnesium, vitamin D, and fluoride. National Academy Press, Washington, D. C., 1997, pp. 288–313.
4) 厚生労働省健康局総務課生活習慣病対策室調査係：日本人の食事摂取基準(2005年版)(概要)．医歯薬出版，東京，2005，1-14頁．
5) Murakami T, Narita N, Nakagaki H et al.: Fluoride intake in Japanese children aged 3–5 years by the duplicate-diet technique. Caries Res 36: 386–390, 2002.
6) Nohno K, Sakuma S, Koga H et al.: Fluoride intake from food and liquid in Japanese children living in two areas with different fluoride concentrations in the water supply. Caries Res 40: 487–493, 2006.
7) Tomori T, Koga H, Maki Y et al.: Fluoride analysis of foods for infants and estimation of daily fluoride intake. Bull Tokyo Dent Coll 45: 19–23, 2004.
8) McClure FJ: Ingestion of fluoride and dental caries. Quantitative relations based on food and water requirements of children one to twelve years old. Am J Dis Child 66: 362–369, 1943.
9) Ophaug RH, Singer L, Harland BF: Estimated fluoride intake of average two-year-old children in four dietary regions of the United States. J Dent Res 59: 777–781, 1980.
10) Ophaug RH, Singer L, Harland BF: Dietary fluoride intake of 6-month and 2-year-old children in four dietary regions of the United States. Am J Clin Nutr 42: 701–707, 1985.
11) Dabeka RW, Mckenzie AD, Conacher HBS et al.: Determination of fluoride in Canadian infant foods and calculation of fluoride intakes by infants. Can J Pub Hlth 73: 188–191, 1982.
12) Featherstone JDB, Shields CP: A study of fluoride intake in New York State residents. Final report. New York State Health Department, Albany, NY, 1988.
13) Dean HD: The investigation of physiological effects by the epidemiological method. Fluorine and dental health. American Association for the Advancement of Science, Washington, D. C., 1942, pp. 23–31.
14) Fejerskov O, Thylstrup A, Larsen MJ: Clinical and structural features and possible pathogenic mechanisms of dental fluorosis. Scand J Dent Res 85: 579–587, 1977.
15) Ekstrand J, Boreus LO, de Chateau P: No evidence of transfer of fluoride from plasma to breast milk. Br Med J 283: 761–762, 1981.
16) Ekstrand J, Spak CJ, Falch J et al.: Distribution of fluoride to human breast milk following intake of high doses of fluoride. Caries Res 18: 93–95, 1984.
17) Gupta S, Seth AK, Gupta A et al.: Transplacental passage of fluorides. J Pediatr 123: 139–141, 1993.
18) Leverett DH, Adair SM, Vaugham BW et al.: Randomized clinical trial of the effect of prenatal fluoride supplements in preventing dental caries. Caries Res 31: 174–179, 1997.

Current Topic

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Caries Ecology Revisited: Microbial Dynamics and the Caries Process

N. Takahashi[a] B. Nyvad[b]

[a]Division of Oral Ecology and Biochemistry, Department of Oral Biology, Tohoku University Graduate School of Dentistry, Sendai, Japan; [b]School of Dentistry, Faculty of Health Sciences, University of Aarhus, Aarhus, Denmark

Key Words

Actinomyces · Dental biofilm · Dental caries · Dental plaque · Ecology · Mutans streptococci · Non-mutans bacteria · Non-mutans streptococci

Abstract

In this essay we propose an extension of the caries ecological hypothesis to explain the relation between dynamic changes in the phenotypic/genotypic properties of plaque bacteria and the demineralization/remineralization balance of the caries process. Dental plaque represents a microbial ecosystem in which non-mutans bacteria (mainly non-mutans streptococci and *Actinomyces*) are the key microorganisms responsible for maintaining dynamic stability on the tooth surface (dynamic stability stage). Microbial acid adaptation and subsequent acid selection of 'low-pH' non-mutans bacteria play a critical role for destabilizing the homeostasis of the plaque by facilitating a shift of the demineralization/remineralization balance from 'net mineral gain' to 'net mineral loss' (acidogenic stage). Once the acidic environment has been established, mutans streptococci and other aciduric bacteria may increase and promote lesion development by sustaining an environment characterized by 'net mineral loss' (aciduric stage). Hence, high proportions of mutans streptococci and/or other aciduric bacteria may be considered biomarkers of sites of particularly rapid caries progression. This cascade of events may change the surface texture of caries lesions from smooth to rough (enamel) or hard to soft (dentin). These clinical surface features can be reversed at any stage of lesion development provided that the acidogenic/aciduric properties of the biofilm are resolved. From an ecological point of view it is therefore not only important to describe which bacteria are involved in caries, but also to know what the bacteria are doing.

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The dental biofilm supports a 'micro-ecosystem' of bacteria that exhibit a variety of physiological characteristics. In particular, the production of acid resulting from sugar metabolism by these bacteria and the subsequent decrease in environmental pH is responsible for demineralization of the tooth surface and formation of dental caries [for review, see Marsh and Nyvad, 2008].

Much research has suggested that mutans streptococci (MS) are the major pathogens of human dental caries. This is because, first, MS are frequently isolated from cavitated caries lesions; second, MS induce caries formation in animals when fed a sucrose-rich diet; third, MS are highly acidogenic and aciduric [Hamada and Slade, 1980; Loesche, 1986], and fourth, MS are able to produce water-insoluble glucan, which promotes bacterial adhesion to the tooth surface and to other bacteria [Hamada and Slade, 1980]. A systematic literature review by Tanzer et al. [2001] confirms a central role of the MS in the initiation of dental caries on enamel and root surfaces.

However, some recent studies indicate that the relationship between MS and caries is not absolute: high proportions of MS may persist on tooth surfaces without le-

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Nobuhiro Takahashi
Division of Oral Ecology and Biochemistry, Department of Oral Biology
Tohoku University Graduate School of Dentistry
4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575 (Japan)
Tel. +81 22 717 8294, Fax +81 22 717 8297, E-Mail nobu-t@mail.tains.tohoku.ac.jp

sion development, and caries can develop in the absence of these species [Nyvad, 1993; Bowden, 1997; Aas et al., 2008]. Under such circumstances, it is suggested that acidogenic and aciduric bacteria other than MS, including 'low-pH' non-MS and *Actinomyces* [van Houte et al., 1994, 1996; Sansone et al., 1993] are responsible for the initiation of caries. Recent molecular analyses have strengthened this concept by showing that the microflora associated with white spot lesions is more diverse than hitherto appreciated and that novel phylotypes and species including *A. gernesceriae, A. naeslundii,* and *A. israelii* as well as a broad range of non-MS and *Veillonela* spp. may also play a role [Becker et al., 2002; Aas et al., 2008]. Since all the bacteria that have been associated with caries belong to the normal microflora of the oral cavity, dental caries has been described an endogenous infection [Fejerskov and Nyvad, 2003]. Endogenous infections may occur when members of the resident flora obtain a selective advantage over other species whereby the homeostatic balance of the biofilm is disturbed [Marsh and Martin, 1999]. Therefore, an ecological hypothesis is attractive [Marsh, 1994].

Concurrently with these changes in the interpretation of the microbial etiology of caries, novel concepts have evolved around the caries process itself. Thus, there is a growing awareness that caries lesions can be managed by non-operative interventions [Fejerskov, 1997; Fejerskov et al., 2008]. Moreover, it has been demonstrated that the effect of such interventions is reflected in the clinical appearance and activity of the lesions [Nyvad and Fejerskov, 1997; Nyvad et al., 2003, 2005; Thylstrup et al., 1994].

So far, these clinical and microbiological advances have not been integrated into a comprehensive concept that may broaden our understanding of caries. Given these circumstances, the aim of this paper is to revisit the 'ecological plaque hypothesis' pioneered by Carlsson [1986] and Marsh [1994, 2003] and to clarify the relationship between the ecological succession of bacteria in dental plaque and the caries process.

Recent Concepts of the Caries Process

Dental caries has been described as a chronic disease that progresses slowly in most individuals. The disease is seldom self-limiting and, in the absence of treatment, caries progresses until the tooth is destroyed. The localized destruction of the hard tissues, often referred to as the lesion, is the sign or symptom of the disease [Fejerskov et al., 2008]. Lesion progression is often depicted on a linear scale ranging from initial loss of mineral at the ultrastructural level to total destruction of the tooth. In reality, however, caries lesion development is a highly dynamic series of processes with alternating periods of progression and arrest/regression [Backer-Dirks, 1966; Nyvad et al., 2003]. Lesion progression may be arrested at any stage of lesion development, even at the stage of frank cavitation [Lo et al., 1998], provided the local environmental conditions, e.g. biofilm control and topical fluoride exposure, are favorable [Nyvad and Fejerskov, 1997]. Hence, the clinical stages of caries represent nothing but historical signs of past caries experience. What may be perceived clinically as an 'incipient' or 'early' lesion may in reality turn out to be an 'aged' established lesion that has been present in the oral cavity for months or years. Likewise, carious cavities may have experienced major differences in their history in the oral cavity.

Changes in the progression rate of caries are associated with alterations of the surface features of the lesions, active non-cavitated enamel lesions being dull and rough and inactive non-cavitated enamel lesions being shiny and smooth [for review, see Thylstrup et al., 1994]. These clinical distinctions have been shown to provide a reliable and valid classification of caries lesion activity [Nyvad et al., 1999, 2003]. Furthermore, such classification has offered novel information about caries lesion transition patterns [Baelum et al., 2003; Lima et al., 2008] and served as a useful basis of selecting high-risk patients [Hausen et al., 2007] in randomized clinical trials. Therefore, when trying to understand the clinical dynamics of caries, assessment of the surface texture of lesions may be a more sensitive parameter than merely assessing the stage of severity of a lesion as revealed by the presence or absence of a cavity.

From a biochemical point of view, the caries process is much more complex. Metabolic processes are constantly taking place in the dental plaque as a result of microbial activity, and this is reflected by continuous, rapid fluctuations in plaque pH, both when the plaque is starved and fed [Newman et al., 1979]. Hence, any clinically sound or carious tooth surface that is covered by an undisturbed plaque may experience minute mineral losses and mineral gains depending on the metabolic status of the microflora. The key point is that only when the cumulative result of the de- and remineralization processes produces a net mineral loss over time may a caries lesion develop or progress [Manji et al., 1991]. Such situations are likely to occur when there is a drift of pH in the biofilm, e.g. as a consequence of increased carbohydrate availability or reduced salivary clearance. By contrast,

when the integrated de- and remineralization processes result in a net mineral gain over time, this may lead to deposition of minerals in the tooth surface and arrest of lesion development. This explains why the caries process has been regarded as a ubiquitous and natural phenomenon [Manji et al., 1991]. Because of the constantly metabolically active biofilm, these processes cannot be prevented, but they can be controlled to the extent that caries does not appear clinically [Fejerskov, 1997; Kidd and Fejerskov, 2004]. This new microdynamic concept of caries suggests that an updated explanation of the microbial ecology of caries must take into consideration that the caries activity may change over time in response to pH drifts in the biofilm.

Microbial Characteristics and the Caries Process

Distribution of MS and Non-Mutans Bacteria in Supragingival Dental Biofilm at Clinically Healthy Sites and in Carious Lesions

In situ studies have shown that the initial colonizers of newly cleaned tooth surfaces constitute a highly selected part of the oral microflora, mainly *S. sanguinis*, *S. oralis* and *S. mitis* 1 [Nyvad and Kilian, 1987]. Together, these three streptococcal species may account for 95% of the streptococci and 56% of the total initial microflora [Li et al., 2004; Nyvad and Kilian, 1987]. Surprisingly, MS comprise only 2% or less of the initial streptococcal population, irrespective of the caries activity of the individual [Nyvad and Kilian, 1990a]. These observations imply that the vast majority of the early colonizers on teeth belong to the 'mitis group'. These bacteria as well as other viridans group streptococci, except for the MS, are often referred to as the non-MS, which are genetically distinguished from the MS that belong to the 'mutans group' [Kawamura et al., 1995]. As the microflora ages it shifts from *Streptococcus*-dominant to *Actinomyces*-dominant [Syed and Loesche, 1978; van Palenstein Helderman, 1981]. The predominant species in mature smooth surface plaque belong to *Actinomyces* and *Streptococcus*, most of which are non-MS [Ximénez-Fyvie et al., 2000]. MS are found in very low numbers [Bowden et al., 1975].

The proportion of MS in plaque covering white spot lesions in enamel is often higher than at clinically healthy sites, although still rather low, ranging between 0.001 and 10% [van Houte et al., 1991b]. Meanwhile, non-MS and *Actinomyces* still remain major bacterial groups in enamel lesions. In fact, it has been shown that in the absence of MS and lactobacilli, the initial dissolution of enamel can be induced by members of the early microflora, exclusively [Boyar et al., 1989].

In cavitated lesions in dentine, including rampant caries, MS constitute about 30% of the total flora [Boue et al., 1987; Loesche et al., 1984; Milnes and Bowden, 1985], indicating that these species are associated with progressive stages of caries. By contrast, MS are encountered less frequently at the advancing front of dentin caries where lactobacilli, prevotellae and *Bifidobacterium* are more prevalent [Aas et al., 2008; Becker et al., 2002; Chhour et al., 2005; Edwardsson, 1974; Munson et al., 2004].

Non-MS as Generalists and MS as Specialists: How Non-MS Can Become Dominant in Supragingival Plaque

Most non-MS have adhesins [Gibbons, 1989; Kolenbrander, 2000] which adhere to proteins and sugar chains of acquired pellicles coating the tooth surface. This seems to be one of the reasons for the dominance of non-MS at the initial stage of plaque formation. In addition, most oral streptococci produce extracellular polysaccharides such as glucans and fructans [Banas and Vickerman, 2003; Whiley and Beighton, 1998]. Polysaccharides can fill the gaps between bacteria and form the matrix of plaque, and accelerate plaque formation.

On the other hand, MS do not attach efficiently to the acquired pellicle [Nyvad and Kilian, 1990b], although they have adhesins such as the antigen I/II. Instead, these bacteria have been emphasized to produce water-insoluble glucans, which are adhesive and capable of accelerating bacterial accumulation. However, it should be noted that glucans only act as additional factors in plaque formation, and that not only MS but also non-MS can produce glucans [Banas and Vickerman, 2003; Vocca-Smith et al., 2000].

Both non-MS and MS metabolize various sugars and produce acids. When sugar is supplied in excess, streptococci can store the extra sugars as intracellular polysaccharides (IPS) [Hamilton, 1976; Takahashi et al., 1991; van Houte et al., 1970], and they can utilize the IPS as an energy source to produce acids when sugar is limited as occurs between meals. The final pH values of non-MS when grown with sugars are heterogeneous, ranging from 4 to 5.2, whereas those of MS are more homogeneous, being around 4 [Hardie, 1986]. In general, on the basis of final pH values, MS are more acidogenic and aciduric than non-MS. It should be realized, however, that the final pH values of non-MS can be much lower than pH 5.5 [Hardie, 1986], the 'critical' pH for the demineralization of enamel.

Non-MS have a variety of extracellular glycosidases [Whiley and Beighton, 1998] that can liberate sugars and amino-sugars from glycoproteins such as the mucin contained in saliva. Furthermore, all non-MS grow on amino-sugars [Byers et al., 1996; Whiley and Beighton, 1998]. This is an advantage for non-MS in the oral cavity, where salivary glycoproteins are always available.

In addition, most non-MS can utilize arginine or arginine-containing peptides available in saliva through the arginine deiminase system, which degrades the arginine molecule to ammonia and carbon dioxide with production of ATP. Overall, this metabolic pathway produces alkali and neutralizes the intracellular and the environmental pH [Burne and Marquis, 2000]. Arginine deiminase system is helpful for non-MS not only to utilize arginine as an energy source but also to survive under the acidic conditions in the oral cavity. However, most MS do not have these metabolic features.

In summary, non-MS have diverse physiological activities, suggesting that they are generalists, versatile enough to adapt to various conditions in supragingival biofilm, and this could be the reason why they are the dominant streptococci in supragingival biofilm. On the other hand, MS are aciduric specialists in sugar metabolism and acid production, which make them less competitive in clinically sound supragingival environments.

Acidogenicity and Acidurance of Non-MS: Key Factors in the Caries Process

It is clear that an ability both to produce acid (acidogenicity) and to tolerate a low-pH environment (acidurance) is a crucial feature for microorganisms responsible for caries. Sansone et al. [1993] compared the microbial composition of dental plaque at clinically healthy sites and white spot lesions and found that non-MS were dominant at both sites while MS were present at low and similar levels at both sites. However, the ability of plaque to reduce pH in vitro was significantly greater at white spot lesions (pH 4.13) than at clinically healthy sites (pH 4.29). These results suggest that MS are neither a unique causative agent for white spot lesions, nor a main determinant of the acidogenicity of plaque.

In order to evaluate the acidogenicity of the non-MS, Sansone et al. [1993] further grew these bacteria in liquid culture media supplemented with 1% glucose and measured the final pH of the culture media. In agreement with Svensäter et al. [2003], they found that non-MS are heterogeneous for acidogenicity: some strains lowered the culture pH to below 4.4, a pH comparable to that produced by MS, whereas for other strains the pH-lowering capacity was less pronounced. In addition, the proportion of acidogenic non-MS was higher at white spot lesions than at clinically healthy sites. The acidogenic non-MS, identified as *S. gordonii*, *S. oralis*, *S. mitis* and *S. anginosus*, were subsequently designated as 'low-pH' non-MS [van Houte, 1994], and it was suggested that the pH-lowering capacity of plaque may be related to the proportion of 'low-pH' non-MS [van Houte et al., 1991a, 1991b]. Later observations by van Ruyven et al. [2000] have supported this notion.

The question still remains: Which of the non-MS are to be considered 'low-pH' non-MS? Alam et al. [2000] obtained two groups of *S. oralis* – one comprised the total *S. oralis* population in dental plaque, whereas the other comprised aciduric strains that were able to grow at pH 5.2. They then differentiated these strains into 15 genotypes on the basis of genetic similarity. The distributions of genotypes were different between the total bacterial group and the aciduric group; only some genotypes of *S. oralis* seemed to be aciduric and to form an aciduric subpopulation. These results are in line with another study showing that strains of non-MS differ distinctly by their rate of acid production at decreasing pH; in particular some strains within *S. mitis* 1, *S. oralis* and *S. gordonii* are capable of producing acids as rapidly as many *S. mutans* strains at pH 5.0 and 5.5 [de Soet et al., 2000]. Collectively, it is suggested that the group of 'low-pH' non-MS comprise a mosaic of acidogenic subpopulations of each species of non-MS.

Involvement of Actinomyces

Most of our knowledge about the role of *Actinomyces* in caries stems from studies of root surface caries. However, there is no evidence that *Actinomyces* spp. have a specific role in root caries. In fact, a review of the literature has concluded that the basic patterns of microbial colonization are identical on enamel and root surfaces, structurally as well as microbiologically [Nyvad, 1993].

As with enamel caries, MS comprise only a small proportion of the microflora of root surface caries lesions. van Houte et al. [1996] reported that non-MS and *Actinomyces* spp. were dominant in dental plaque covering root surface caries and that the isolated *Actinomyces* strains were heterogenous with respect to acidogenicity: strains isolated from root surface caries were more acidogenic than those from clinically healthy root surfaces. Meanwhile, Brailsford et al. [2001] observed that, in subjects with root surface caries, aciduric bacteria able to grow at pH 4.8 comprised 21.6% of the total microflora in root surface caries lesions (lactobacilli and *Actinomyces* were

dominant), whereas aciduric bacteria comprised 10.7% in clinically sound root surfaces (*Actinomyces* dominant). However, in subjects without root surface caries, aciduric bacteria comprised only 1.4% of total microflora in clinically sound root surfaces. These findings indicate an association between acidogenic/aciduric *Actinomyces*, i.e. 'low-pH' *Actinomyces* and root surface caries.

Actinomyces are as versatile to adapt to the dental biofilm environment as are the non-MS; they have adhesin-mediated adhesion to tooth surfaces, produce acids from various sugars, and synthesize intracellular and extracellular polysaccharides. In addition, *Actinomyces* have a unique glycolytic system [Takahashi et al., 1995] in which they utilize high-energy polyphosphate and pyrophosphate compounds for synthesis of hexokinase and phosphofructokinase, respectively, acting as phosphoryl donors instead of ATP. This means that *Actinomyces* are able to exploit a surplus ATP to synthesize polyphosphate as an energy reservoir, and salvage energy from pyrophosphate, a high-energy-phosphoryl-bond-containing byproduct from the metabolism of polymers such as nucleic acids and glycogens. In addition, *Actinomyces* are often ureolytic [Kleinberg, 2002; Yaling et al., 2006] and can utilize lactate as a carbon source for growth [Takahashi et al., 1996]. These diverse physiological characteristics of *Actinomyces* seem to be advantageous to survive and dominate in supragingival plaque [Takahashi and Yamada, 1999b].

Acid Adaptation and Acid Selection: Adaptive Changes in Acidurance and Acidogenicity and the Consequent Selection of 'Low-pH' Non-MS

Non-MS are not only genotypically heterogenous, but they are also able to change their physiological characteristics adaptively. Takahashi and Yamada [1999a] have shown that when these bacteria were exposed to an acidic environment, they increased their acidogenicity. These bacteria were grown first at pH 7.0 and afterwards at pH 5.5 for a short time: 30, 60 and 90 min. The bacteria were then harvested, washed and incubated with glucose, and the final pH values were measured as a marker of acidogenicity. Their acidogenicity or final pH values varied (pH 4.04–4.33), but after incubation at pH 5.5 for 60 min, all the bacteria increased their acidogenicity (pH 3.93–4.12).

Non-MS were also able to increase their acidurance adaptively [Takahashi and Yamada, 1999a]. Bacteria initially grown at pH 7.0 were killed by acid stress in a strain-dependent manner following exposure to pH 4.0 for 60 min (survival rate: 0.0009–71%), but after pre-acidification at pH 5.5 for 60 min, all the bacteria increased their acidurance (survival rate: 0.4–81%).

The biochemical mechanisms underlying the acid adaptation are considered to involve the following mechanism [Quivey et al., 2000]: (1) an increase in proton impermeability of the cell membrane; (2) induction of proton-translocating ATPase (H^+-ATPase) activity that expels proton from cells; (3) induction of the arginine deiminase system that produces alkali from arginine or arginine-containing peptides, and (4) induction of stress proteins that protect enzymes and nucleic acids from acid denaturation. In non-MS, the increase in activities of H^+-ATPase and arginine deiminase and expression of stress proteins (homologues of heat shock protein, Hsp60 and Hsp70) were observed following incubation at pH 5.5 [Takahashi and Yamada, 1999a].

In the oral cavity, acidification of the biofilm due to frequent sugar intake or poor salivary secretion can be a driving force to enhance the acidogenicity and acidurance of the non-MS, resulting in establishment of a more acidic environment. Even if acid adaptation occurs, non-MS are still so heterogeneous with respect to acidurance that the population of more aciduric strains, i.e. 'low-pH' non-MS will increase selectively in this environment. This will cause a shift in the composition and acidogenic potential of the biofilm, which, provided the demineralization/remineralization balance is disturbed over an extended period of time, leads to dental caries. Similar microbial acid adaptation and acid selection processes may occur in *Actinomyces*.

Competition between Non-MS and MS

Transient Acidification. Although 'low-pH' non-MS can adaptively increase their acidurance and acidogenicity, and take over the position in supragingival plaque, MS are more competitive under severely acidic conditions. Following a rapid exposure to pH 4.0 for 60 min as often observed in dental plaque after a sugar exposure, *S. sanguinis* ATCC 10556, a strain of 'low-pH' non-MS, was able to survive. However, this bacterium temporarily lost the ability to grow, along with the inactivation of glycolytic enzymes, and did not start growing again until 90 min after the pH had returned to neutral [Takahashi et al., 1997]. By contrast, the growth of *S. mutans* NCTC 10449 at pH 4.0 was not influenced at all. In view of this observation, it is expected that the population of non-MS decreases gradually during frequent acidification, whereas the proportion of MS would increase.

Prolonged Acidification. Experiments using in vitro cultures of mixtures of oral bacterial species have clearly

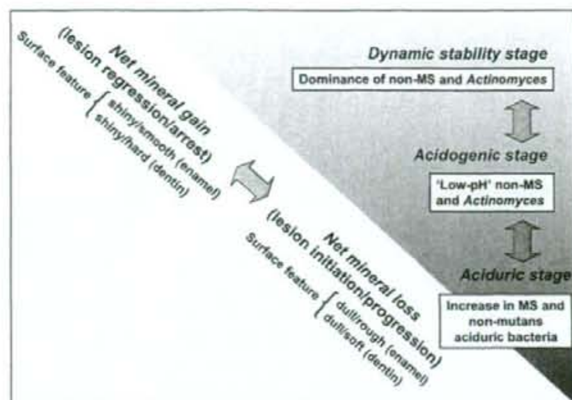

Fig. 1. An extended caries ecological hypothesis explaining the relationship between acidogenic and aciduric shifts in the composition of the dental biofilm and changes in the mineral balance of the dental hard tissues. Note that the cascade of ecological events in the biofilm is reversible and is reflected in the surface features of the dental hard tissues at any stage of lesion formation. MS = Mutans streptococci. For detailed explanation, see text.

shown that prolonged acidification is the driving force behind the emergence of MS in dental plaque. Bowden and Hamilton [1987] demonstrated that *S. sanguinis* (formerly *S. sanguis*) was dominant when the pH was kept at 7.0–6.0 in a mixed continuous culture, whereas when pH was shifted to 5.5, *S. mutans* overcame *S. sanguinis*, although *S. sanguinis* survived in the culture at pH 4.5. A similar phenomenon was observed by Bradshaw and Marsh [1998]. They established a continuous culture with 9 oral bacterial species and demonstrated that non-MS (*S. oralis* and *S. gordonii*) were dominant when the pH was kept at pH 7.0 during daily pulses of glucose for 10 days, whereas when the pH was allowed to fall to a preset value of 5.0, MS as well as lactobacilli became dominant; non-MS were excluded from the consortium when pH was allowed to fall without control (final pH = 3.83). Similarly, Takahashi et al. [1997] showed that a strain of 'low-pH' non-MS (*S. sanguinis* ATCC 10556) was not able to grow at pH $\leq$ 4.2 in a complex liquid medium under anaerobic conditions, while *S. mutans* was still able to grow at pH 4.2. Given these observations, it is suggested that prolonged acidic conditions around pH 5.5 may cause the emergence of MS in the microbial flora and that more severe acidic conditions around pH 4 may exclude the non-MS. In the oral cavity, prolonged acidic conditions (pH $\leq$ 5.5) can occur in carious cavities [Dirksen et al., 1962; Hojo et al., 1994], where clearance of acids is hampered. This may be the reason why MS and particularly lactobacilli are frequently isolated from established carious cavities.

An Extended Caries Ecological Hypothesis

In the light of the foregoing we suggest an extended caries ecological hypothesis that explains the relationship between the composition of the dental plaque and the caries process (fig. 1). In this hypothesis, dental plaque is a dynamic microbial ecosystem in which non-mutans bacteria such as non-MS and *Actinomyces* are the key players for maintaining dynamic stability. These bacteria can produce acids from sugary foods and the resulting acids can demineralize the enamel. However, the temporary decreases in pH are easily returned to neutral level by homeostatic mechanisms in the plaque [Marsh and Martin, 1999]. This is a natural pH cycle, which occurs numerous times daily in supragingival plaque (dynamic stability stage).

However, when sugar is supplied frequently or salivary secretion is too scarce to neutralize the acids produced, the pH decreases in the plaque may enhance the acidogenicity and aciduranсe of the non-mutans bacteria adaptively. Under such conditions the population of the 'low-pH' non-MS and *Actinomyces* then increases via acid selection, leading to a microbial shift to a more acidogenic microflora. These changes in the phenotype and genotype of the microflora may shift the demineralization/remineralization balance from 'net mineral gain' to 'net mineral loss' and initiate lesion development (acidogenic stage). At this stage, lesion development could also be arrested with de-adaptation of the microflora, provided that the mineral balance is restored to a 'net mineral gain' by reduced environmental acidification.

If prolonged acidic environments prevail, lesion development ('net mineral loss') is likely to progress. In these environments, more aciduric bacteria such as MS and lactobacilli may replace the 'low-pH' non-mutans bacteria and further accelerate the caries process (aciduric stage). However, even at this highly aciduric stage, the mineral balance and composition of the microflora could possibly be reversed by modification of the acidic environment, e.g. as a result of sugar restriction [de Stoppelaar et al., 1970].

In this scenario, the microbial acid adaptation and the subsequent acid selection of 'low-pH' non-mutans bacteria play a crucial role in destabilizing the homeostasis of

the biofilm and facilitating lesion development. Moreover, once the acidic environment has been established, the proportion of aciduric bacteria such as MS and lactobacilli may increase and act as promoters of lesion progression by sustaining an environment characterized by 'net mineral loss'. Hence, high proportions of MS and/or other aciduric bacteria may be considered biomarkers of sites that undergo particularly rapid caries development [Bowden et al., 1976; Chhour et al., 2005; Macpherson et al., 1990; Nyvad and Kilian, 1990b]. We suggest that this cascade of events is associated with changes in the surface texture of the dental hard tissues from smooth to rough (enamel) or from hard to soft (dentin) [Nyvad et al., 1999, 2003].

Two decades ago, Carlsson [1986] presented a caries microbiological hypothesis by which he speculated that ecological changes in the oral flora were determined by competition for nutrients. Carlsson proposed that at low levels of sugars, the oral microflora would be dominated by bacteria with a high affinity for sugars (the 'gleaners'), whereas at consistently higher concentrations of sugars, bacteria with lower affinity for sugars, but with high growth rates, would be favored (the 'exploiters'). Under the latter condition the metabolic end products established acidic environments favoring an outgrowth of aciduric bacteria, the so-called 'pH-strategists' [Carlsson, 1986]. This concept was further developed as the ecological plaque hypothesis by Marsh [1994, 2003], who focused on the dynamic and reversible processes of de- and remineralization in the plaque by linking between sugar supply, pH change and microflora shift. We suggest that the ecological concept of caries should be extended and strengthened by including clinical manifestations of caries lesion processes, and by detailing the microbial acid adaptation and acid selection processes.

Clinical and Scientific Perspectives

The extended caries ecological hypothesis supports the 'mixed-bacteria ecological approach' proposed by Kleinberg [2002] that the proportion of acid- and base-producing bacteria is the core of caries activity. Clearly, the extended hypothesis undermines the view that dental caries is a classical infectious disease, and therefore that prevention and control of this condition by elimination of a specific group of microorganisms, such as the MS, through vaccination, gene therapy or antimicrobial treatment, is unwise. Rather environmental control of the microflora should be achieved by stimulating the non-mutans bacteria such as non-MS and *Actinomyces* by avoiding acidification of the dental biofilm.

Practical solutions to this strategy may include mechanical plaque control, reduction/substitution of the intake of sugary foods and/or application of pH-neutralizing techniques such as saliva stimulation. Even if the effect of such interventions on the composition of the microflora is sparsely documented, it has been shown that dietary modification may facilitate such changes. Hence, de Stoppelaar et al. [1970] observed a clear reduction in the proportion of MS at carious and filled sites at the expense of *S. sanguis* following a 3-week period of sucrose restriction. These changes were reversed when individuals resumed a normal diet containing sucrose. Conventional culture studies of young dental plaque in caries-inactive individuals have failed to reveal a consistent microbial response pattern to sucrose-regulated diets [Staat et al., 1975; Scheie et al., 1984]. In these studies, sucrose-related modulation of the microflora was found to depend on prior oral colonization by mutans streptococci, and these species were not entirely eliminated on a low-sucrose diet. It is interesting to speculate that differences in the propagation of MS might reflect differences in acid tolerance between clones of these species [Welin-Neilands and Svensäter, 2007]. Future studies describing the site-specific microbial shifts in response to sucrose should therefore focus on both the MS and the non-mutans bacteria, e.g. by applying molecular identification methods.

An important consequence of the extended hypothesis is that knowledge about the acidogenic and aciduric properties of bacteria, i.e. the phenotypic characteristics, and their regulatory mechanism may be a more relevant parameter than knowledge about their taxonomy. The phenotypes of most bacteria have already been well described in textbooks such as the Bergey's Manual of Systematic Bacteriology [Holt, 1984]. Nevertheless, such descriptions are not particularly helpful to explain the in vivo behaviors since bacterial phenotypic characteristics may change depending on the local environmental conditions. Therefore, from an ecological point of view it is not only important to describe which bacteria are involved in caries but also to know what the bacteria are doing [Takahashi, 2005].

Recently, van Ruyven et al. [2000] have detected non-mutans aciduric bacteria other than non-MS and *Actinomyces* from dental biofilms covering white spot lesions. They found that these bacteria consisted of various species including lactobacilli and *Bifidobacterium*. Interestingly, the samples differed with respect to dominance of

particular bacterial species, suggesting that any bacterial species can participate in the development of caries as long as they are aciduric and dominant [Bowden, 1984]. In this essay we have focused on the non-MS and the *Actinomyces* as the major non-mutans aciduric bacteria because detailed studies have been conducted for these bacteria. However, it would not be surprising if other non-mutans aciduric bacteria were found to be associated with dental caries. As stated above, it is not the genotype per se, but the phenotype in a certain environment, i.e. the acidogenic and aciduric potential of the bacteria, that is conducive to a microbial shift leading to caries.

According to the extended hypothesis, there is a firm association between the de- and remineralization balance of caries lesions and the overall composition of the microflora. In the in situ study of Nyvad and Kilian [1990b], root surface caries lesions experiencing the highest mineral loss, as assessed by quantitative microradiography, were dominated by uniform *Actinomyces* spp., or a combination of MS and *Lactobacillus* spp., whereas lesions experiencing a smaller mineral loss were associated with a more diverse microbiota including non-MS, MS, *Actinomyces*, lactobacilli, *Bifidobacterium* as well as lactate-metabolizing species (*Veillonella* spp.). Such differences in the pattern of the microflora in response to different lesion progression rates not only lend support to the suggested acidogenic and aciduric stages of bacterial succession in caries, but also conform with the concept that microbial diversity may exert a protective effect on the dynamic stability of the biofilm community, recently referred to as the 'insurance hypothesis' [Yachi and Loreau, 1999; Boles et al., 2004]. Therefore, in the future, if we truly wish to advance the ecological understanding of caries, it is important to describe the total microbiota of caries lesions by studying lesions with a known age and history in the oral cavity or, alternatively, employ clinical caries diagnostic methods that reflect the activity state of lesions [Nyvad et al., 1999, 2003].

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References

Aas JA, Griffen AL, Dardis SR, Lee AM, Olsen I, Dewhirst FE, Leys EJ, Paster BJ: Bacteria of dental caries in primary and permanent teeth in children and young adults. J Clin Microbiol 2008;46:1407–1417.

Alam S, Brailsford SR, Adams S, Allison C, Sheehy E, Zoitopoulos L, Kidd EA, Beighton D: Genotypic heterogeneity of *Streptococcus oralis* and distinct aciduric subpopulations in human dental plaque. Appl Environ Microbiol 2000;66:3330–3336.

Backer-Dirks O: Posteruptive changes in dental enamel. J Dent Res 1966;45:503–511.

Baelum V, Machiulskiene V, Nyvad B, Richards A, Vaeth M: Application of survival analysis to caries lesion transitions in intervention trials. Community Dent Oral Epidemiol 2003;31:252–260.

Banas JA, Vickerman MM: Glucan-binding proteins of the oral streptococci. Crit Rev Oral Biol Med 2003;14:89–99.

Becker MR, Paster BJ, Leys EJ, Moeschberger ML, Kenyon SG, Galvin JL, Boches SK, Dewhirst FE, Griffen AL: Molecular analysis of bacterial species associated with childhood caries. J Clin Microbiol 2002;40:1001–1009.

Boles BR, Thoendel M, Singh PK: Self-generated diversity produces 'insurance effects' in biofilm communities. Proc Natl Acad Sci USA 2004;101:16630–16635.

Boue D, Armau E, Tiraby G: A bacteriological study of rampant caries in children. J Dent Res 1987;66:23–28.

Bowden GH: Possibilities for modifying the caries attack by altering the oral microflora. J Can Dent Assoc 1984;50:169–172.

Bowden GH: Does assessment of microbial composition of plaque/saliva allow for diagnosis of disease activity of individuals? Community Dent Oral Epidemiol 1997;25:76–81.

Bowden GH, Hamilton IR: Environmental pH as a factor in the competition between strains of the oral streptococci *Streptococcus mutans*, *S. sanguis*, and '*S. mitior*' growing in continuous culture. Can J Microbiol 1987;33: 824–827.

Bowden GH, Hardie JM, McKee AS, Marsh PD, Fillery ED, Slack GL: The microflora associated with developing carious lesions of the distal surfaces on the upper first premolars in 13- to 14-year-old children; in Stiles HM, Loesche WJ, O'Brien TC (eds): Microbial Aspects of Dental Caries. Washington, IRL Press, 1976, vol 1, pp 223–241.

Bowden GH, Hardie JM, Slack GL: Microbial variations in approximal dental plaque. Caries Res 1975;9:253–277.

Boyar RM, Thylstrup A, Holmen L, Bowden GH: The microflora associated with the development of initial enamel decalcification below orthodontic bands in vivo in children living in a water-fluoridated area. J Dent Res 1989; 68:1734–1738.

Bradshaw DJ, Marsh PD: Analysis of pH-driven disruption of oral microbial communities in vitro. Caries Res 1998;32:456–462.

Brailsford SR, Shah B, Simons D, Gilbert S, Clark D, Ines I, Adams SE, Allison C, Beighton D: The predominant aciduric microflora of root-caries lesions. J Dent Res 2001;80:1828–1833.

Burne RA, Marquis EM: Alkali production by oral bacteria and protection against dental caries. FEMS Microbiol Lett 2000;193:1–6.

Byers HL, Homer KA, Beighton D: Utilization of sialic acid by viridans streptococci. J Dent Res 1996;75:1564–1571.

Carlsson J: Metabolic activities of oral bacteria; in Thylstrup A, Fejerskov O (eds): Textbook of Cariology. Copenhagen, Munksgaard, 1986, pp 74–106.

Chhour KL, Nadkarni MA, Buyn R, Martin FE, Jacques NA, Hunter N: Molecular analysis of microbial diversity in advanced caries. J Clin Microbiol 2005;43:843–849.

de Soet JJ, Nyvad B, Kilian M: Strain-related acid production by oral streptococci. Caries Res 2000;34:486–490.

de Stoppelaar JD, van Houte J, Dirks OB: The effect of carbohydrate restriction on the presence of *Streptococcus mutans* and *Streptococcus sanguis* and iodophilic polysaccharide-producing bacteria in human dental plaque. Caries Res 1970;4:114–123.

Dirksen TR, Little MF, Bibby BG, Crump SL: The pH of carious cavities. 1. The effect of glucose and phosphate buffer on cavity pH. Arch Oral Biol 1962;7:49–58.

Edwardsson S: Bacteriological studies of deep areas of carious dentine. Odontol Revy Suppl 1974;32:1–143.

Fejerskov O: Concepts of dental caries and their consequences for understanding the disease. Community Dent Oral Epidemiol 1997;25: 5–12.

Fejerskov O, Nyvad B: Is dental caries an infectious disease? Diagnostic and treatment consequences for the practitioner; in Schou L (ed): Nordic Dentistry 2003 Yearbook. Copenhagen, Quintessence Publishing Co Ltd, 2003, pp 141–152.

Fejerskov O, Nyvad B, Kidd EAM: Pathology of dental caries; in Fejerskov O, Kidd EAM (eds): Dental Caries. The Disease and Its Clinical Management, ed 2. Oxford, Blackwell Munksgaard, 2008, pp 19–48.

Gibbons RJ: Bacterial adhesion to oral tissue: a model for infectious diseases. J Dent Res 1989;68:750–760.

Hamada S, Slade HD: Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol Rev 1980;44:331–380.

Hamilton IR: Intracellular polysaccharide synthesis by cariogenic microorganisms; in Stiles HM, Loesche WJ, O'Brien TC (eds): Proceedings: Microbial Aspects of Dental Caries (a special supplement to Microbiology Abstracts). New York, Information Retrieval, Inc, 1976, vol 3, pp 683–701.

Hardie JM: Oral streptococci; in Sneath PHA, Mair NS, Sharpe ME, Holt JG (eds): Bergey's Manual of Systematic Bacteriology. Baltimore, Williams & Wilkins, 1986, vol 2, pp 1054–1063.

Hausen H, Seppa L, Poutanen R, Niinimaa A, Lathi S, Kärkkainen S, Pietalä I: Noninvasive control of dental caries in children with active initial lesions. A randomized clinical trial. Caries Res 2007;41:384–391.

Hojo S, Komatsu M, Okuda R, Takahashi N, Yamada T: Acid profiles and pH of carious dentin in active and arrested lesions. J Dent Res 1994;73:1853–1857.

Holt JG (ed): Bergey's Manual of Systematic Bacteriology. Baltimore, Williams & Wilkins, 1984.

Kawamura Y, Hou XG, Sultana F, Miura H, Ezaki T: Determination of 16S rRNA sequences of *Streptococcus mitis* and *Streptococcus gordonii* and phylogenetic relationships among members of the genus *Streptococcus*. Int J Syst Bacteriol 1995;45:406–408.

Kidd EAM, Fejerskov O: What constitutes dental caries? Histopathology of carious enamel and dentin related to the action of cariogenic biofilms. J Dent Res 2004;83(Spec Iss C): C35–C38.

Kleinberg I: A mixed-bacteria ecological approach to understanding the role of oral bacteria in dental caries causation: an alternative to *Streptococcus mutans* and the specific-plaque hypothesis. Crit Rev Oral Biol Med 2002;13:108–125.

Kolenbrander PE: Oral microbial communities: biofilms, interactions, and genetic systems. Annu Rev Microbiol 2000;54:413–437.

Li J, Helmerhorst, Leone CW, Troxler RF, Yaskell T, Haffajee AD, Socrasnsky SS, Oppenheim FG: Identification of early microbial colonizers in human dental biofilm. J Appl Microbiol 2004;97:1311–1318.

Lima TJ, Ribeiro CC, Tenuta LM, Cury JA: Low-fluoride dentifrice and caries lesion control in children with different caries experience: a randomized clinical trial. Caries Res 2008; 42:46–50.

Lo EC, Schwarz E, Wong MC: Arresting dentine caries in Chinese preschool children. Int J Paediatr Dent 1998;8:253–260.

Loesche WJ: Role of *Streptococcus mutans* in human dental decay. Microbiol Rev 1986;50: 353–380.

Loesche WJ, Eklund S, Earnest R, Burt B: Longitudinal investigation of bacteriology of human fissure decay: epidemiological studies on molars shortly after eruption. Infect Immun 1984;46:765–772.

Macpherson LMD, MacFarlane TW, Stephen KW: An intra-oral appliance study of the plaque microflora associated with early enamel demineralization. J Dent Res 1990; 69:1712–1716.

Manji F, Fejerskov O, Nagelkerke NJ, Baelum V: A random effects model for some epidemiological features of dental caries. Community Dent Oral Epidemiol 1991;19:324–328.

Marsh PD: Microbial ecology of dental plaque and its significance in health and disease. Adv Dent Res 1994;8:263–271.

Marsh PD: Are dental diseases examples of ecological catastrophes? Microbiology 2003; 149:279–294.

Marsh PD, Martin VM: Dental plaque; in Marsh PD, Martin VM (eds): Oral Microbiology. Oxford, Wright, 1999, pp 58–81.

Marsh PD, Nyvad B: The oral microflora and biofilms on teeth; in Fejerskov O, Kidd EAM (eds): Dental Caries. The Disease and Its Clinical Management, ed 2. Oxford, Blackwell Munksgaard, 2008, pp 163–187.

Milnes AR, Bowden GH: The microflora associated with developing lesions of nursing caries. Caries Res 1985;19:289–297.

Munson MA, Banerjee A, Watson, TF, Wade WG: Molecular analysis of the microflora associated with dental caries. J Clin Microbiol 2004;42:3023–3029.

Newman P, MacFadyen EE, Gillespie FC, Stephen KW: An in-dwelling electrode for in-vivo measurement of the pH of dental plaque in man. Arch Oral Biol 1979;24:503–507.

Nyvad B: Microbial colonization of human tooth surfaces. APMIS 1993;101(suppl 32):7–45.

Nyvad B, Fejerskov O: Assessing the stage of caries lesion activity on the basis of clinical and microbiological examination. Community Dent Oral Epidemiol 1997;25:69–75.

Nyvad B, Kilian M: Microbiology if the early microbial colonization of human enamel and root surfaces in vivo. Scand J Dent Res 1987; 95:369–380.

Nyvad B, Kilian M: Comparison of the initial streptococcal microflora on dental enamel in caries-active and caries-inactive individuals. Caries Res 1990a;24:267–272.

Nyvad B, Kilian M: Microflora associated with experimental root surface caries in humans. Infect Immun 1990b;58:1628–1633.

Nyvad B, Machiulskiene V, Baelum V: Reliability of a new caries diagnostic system differentiating between active and inactive caries lesions. Caries Res 1999;33:252–260.

Nyvad B, Machiulskiene V, Baelum V: Construct and predictive validity of clinical caries diagnostic criteria assessing lesion activity. J Dent Res 2003;82:117–122.

Nyvad B, Machiulskiene V, Baelum V: The Nyvad criteria for assessment of caries lesion activity; in Stookey G (ed): Proceedings of the 7th Indiana Conference, Indianapolis, Indiana. Clinical Models Workshop: Remin-Demin, Precavitation, Caries. Indianapolis, Indiana University School of Dentistry, 2005, pp 99–116.

Quivey RG Jr, Kuhnert WL, Hahn K: Adaptation of oral streptococci to low pH. Adv Microb Physiol 2000;42:239–274.

Sansone C, van Houte J, Joshipura K, Kent R, Margolis HC: The association of mutans streptococci and non-mutans streptococci capable of acidogenesis at a low pH with dental caries on enamel and root surfaces. J Dent Res 1993;72:508–516.

Scheie AA, Arneberg P, Ørstavik D, Afseth: Microbial composition, pH-depressing capacity and acidogenicity of 3-week smooth surface plaque developed on sucrose-regulated diets in man. Caries Res 1984;18:74–86.

Staat RH, Gawronsky TH, Cressey DE, Harris RS, Folke LEA: Effects of dietary sucrose on the quantity and microbial composition of human dental plaque. J Dent Res 1975;54: 872–880.

Svensäter G, Borgström M, Bowden GHW, Edwardsson S: The acid-tolerant microbiota associated with plaque from initial caries and healthy tooth surfaces. Caries Res 2003;37: 395–403.

Syed SA, Loesche WJ: Bacteriology of human experimental gingivitis: effect of plaque age. Infect Immun 1978;21:821–829.

Takahashi N: Microbial ecosystem in the oral cavity: metabolic diversity in an ecological niche and its relationship with oral diseases; in Watanabe M, Takahashi N, Takada H (eds): Interface Oral Health Science, International Congress Series 1284. Oxford, Elsevier, 2005, pp 103–112.

Takahashi N, Horiuchi M, Yamada T: Effects of acidification on growth and glycolysis of *Streptococcus sanguis* and *Streptococcus mutans*. Oral Microbiol Immunol 1997;12:72–76.

Takahashi N, Iwami Y, Yamada T: Metabolism of intracellular polysaccharide in the cells of *Streptococcus mutans* under strictly anaerobic conditions. Oral Microbiol Immunol 1991;6:299–304.

Takahashi N, Kalfas S, Yamada T: Phosphorylating enzymes involved in glucose fermentation of *Actinomyces naeslundii*. J Bacteriol 1995;177:5806–5811.

Takahashi N, Yamada T: Catabolic pathway for aerobic degradation of lactate by *Actinomyces naeslundii*. Oral Microbiol Immunol 1996;11:193–198.

Takahashi N, Yamada T: Acid-induced acidogenicity and acid tolerance of non-mutans streptococci. Oral Microbiol Immunol 1999a;14:43–48.

Takahashi N, Yamada T: Glucose and lactate metabolism by *Actinomyces naesllundii*. Crit Rev Oral Biol Med 1999b;10:504–518.

Tanzer JM, Livingston J, Thompson AM: The microbiology of primary dental caries in humans. J Dent Educ 2001;65:1028–1037.

Thylstrup A, Bruun C, Holmen L: In vivo caries models – mechanisms for caries initiation and arrestment. Adv Dent Res 1994;8:144–157.

van Houte J: Role of Microorganisms in caries etiology. J Dent Res 1994;73:672–681.

van Houte J, de Moore CE, Jansen HM: Synthesis of iodophilic polysaccharide by human oral streptococci. Arch Oral Biol 1970;15:263–266.

van Houte J, Lopman J, Kent R: The predominant cultivable flora of sound and carious human root surfaces. J Dent Res 1994;73:1727–1734.

van Houte J, Lopman J, Kent R: The final pH of bacteria comprising the predominant flora on sound and carious human root and enamel surfaces. J Dent Res 1996;75:1008–1014.

van Houte J, Sansone C, Joshipura K, Kent R: In vitro acidogenic potential and mutans streptococci of human smooth surface plaque associated with initial caries lesions and sound enamel. J Dent Res 1991a;70:1497–1502.

van Houte J, Sansone C, Joshipura K, Kent R: Mutans streptococci and non-mutans streptococci acidogenic at low pH, and in vitro acidogenic potential of dental plaque in two different areas of the human dentition. J Dent Res 1991b;70:1503–1507.

van Palenstein Helderman WH: Longitudinal microbial changes in developing human supragingival and subgingival plaque. Arch Oral Biol 1981;26:7–12.

van Ruyven FO, Lingstrom P, van Houte J, Kent R: Relationship among mutans streptococci, 'low-pH' bacteria, and iodophilic polysaccharide-producing bacteria in dental plaque and early enamel caries in humans. J Dent Res 2000;79:778–784.

Vocca-Smith AM, Ng-Evans L, Wunder D, Bowen WH: Studies concerning the glucosyltransferase of *Streptococcus sanguis*. Caries Res 2000;34:295–302.

Welin-Neilands J, Svensäter G: Acid tolerance by biofilm cells of mutans streptococci. Appl Environ Microbiol 2007;73:5633–5638.

Whiley RA, Beighton D: Current classification of the oral streptococci. Oral Microbiol Immunol 1998;13:195–216.

Ximénez-Fyvie LA, Haffajee AD, Socransky SS: Microbial composition of supra- and subgingival plaque in subjects with adult periodontitis. J Clin Periodontol 2000;27:722–732.

Yachi S, Loreau M: Biodiversity and ecosystem productivity in a fluctuating environment: the insurance hypothesis. Proc Natl Acad Sci USA 1999;96:1463–1468.

Yaling L, Tao H, Jingyi Z, Xuedong Z: Characterization of the *Actinomyces naeslundii* ureolysis and its role in bacterial acidurity and capacity to modulate pH homeostasis. Microbiol Res 2006;161:304–310.

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Mutual induction of noncollagenous bone proteins at the interface between epithelial cells and fibroblasts from human periodontal ligament

M. Shimonishi[1], J. Hatakeyama[2], Y. Sasano[2], N. Takahashi[3], M. Komatsu[4], M. Kikuchi[1]

[1]Division of Comprehensive Dentistry, Tohoku University Dental Hospital, Sendai, Japan, [2]Division of Craniofacial Development and Regeneration, Department of Craniofacial Engineering and Regeneration, Tohoku University Graduate School of Dentistry, Sendai, Japan, [3]Division of Oral Ecology and Biochemistry, Department of Oral Biology Tohoku University Graduate School of Dentistry, Sendai, Japan, and [4]Division of Operative Dentistry, Department of Restorative Dentistry, Tohoku University Graduate School of Dentistry, Sendai, Japan

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Background and Objective: Epithelial–mesenchymal interactions are responsible for cell differentiation during periodontal regeneration. The present study was undertaken to examine the expression of alkaline phosphatase and noncollagenous bone proteins, such as osteopontin, osteocalcin and bone sialoprotein, with respect to interaction between the cells of the epithelial rests of Malassez and fibroblasts from human periodontal ligament.

Material and Methods: Explants of human periodontal ligament tissues produced outgrowths containing both putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts in a modified serum-free medium. Putative epithelial rests of Malassez cells cultured alone, and human periodontal ligament fibroblasts cultured alone, were used as controls. The expression levels of amelogenin were analyzed by *in situ* hybridization. The expression and distribution of alkaline phosphatase and noncollagenous bone proteins in both cell populations at the interface between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts were analyzed by immunohistochemistry, *in situ* hybridization and reverse transcription-polymerase chain reaction.

Results: Amelogenin mRNA was detected at high levels only in putative epithelial rests of Malassez cells at the interface. Alkaline phosphatase and bone sialoprotein mRNAs were detected significantly at the interface between putative epithelial rests of Malassez cells and human periodontal ligament fibroblast cells. In particular, bone sialoprotein and its mRNA were expressed significantly in human periodontal ligament fibroblasts at the interface between putative epithelial rests of Malassez cells and human periodontal ligament fibroblast cells. The expressions of osteopontin and its mRNA were not different between putative epithelial rests of malassez cells and human periodontal ligament fibroblasts at the interface. Osteocalcin and its mRNA were expressed strongly in putative epithelial rests of

Mitsuru Shimonishi, DDS, PhD, Division of Comprehensive Dentistry, Tohoku University Dental Hospital, 4–1, Seiryo-machi, Aoba-ku, Sendai, 980–8575, Japan
Tel: +81 22 717 8434
Fax: +81 22 717 8434
e-mail: shimo@ddh.tohoku.ac.jp

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Malassez cells at the interface between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts.

Conclusion: These findings indicate that the epithelial–mesenchymal interaction modulates the expression of alkaline phosphatase, osteocalcin and bone sialoprotein in putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts, suggesting that epithelial–mesenchymal interactions play a role in the maintenance of periodontal ligament.

Human periodontal ligament contains the epithelial rests of Malassez, which appear as cellular clusters in cord- or net-like formations or as isolated islands near the cementum. Hertwig's epithelial root sheath cells represent an extension of the epithelium that participates in coronal amelogenesis and which is retained in association with the developing root. Immediately as the outer layer of the dentin matrix starts to calcify, the epithelial cells of the root sheath separate from the dentin surface, and breaks occur in the continuity of its previously continuous double-layered sheet of cells to form the epithelial rests of Malassez. As a result, the epithelial rests of Malassez are odontogenic epithelial cells derived from Hertwig's epithelial root sheath. Amelogenin is the major enamel protein produced by ameloblasts at the differentiation and secretory stages (1). We demonstrated previously that intense immunoreactivity for amelogenin mRNA is observed in putative epithelial rests of Malassez cells, whereas gingival epithelial cells and human periodontal ligament fibroblasts do not show amelogenin mRNA expression (2). Amelogenin is therefore a useful marker for cultured epithelial rests of Malassez cells.

The formation, turnover and repair of mineralized tissues, such as bone, calcified cartilage and teeth, are all complex processes requiring differentiated cell function and extracellular matrix assembly and degradation during normal development as well as during tissue repair after injury. It has been determined that several of the mineral-binding proteins are capable not only of guiding extracellular calcification, but also of mediating cell dynamics, such as cell adhesion, spreading and migration, in mineralized tissues (3–5). The activity of alkaline phosphatase is required to generate the inorganic phosphate needed for hydroxyapatite crystallization. Extracellular inorganic pyrophosphate is a potent inhibitor of hydroxyapatite formation. Alkaline phosphatase hydrolyzes extracellular inorganic pyrophosphate (6–9). Osteopontin, osteocalcin and bone sialoprotein are the major noncollagenous proteins that are secreted by osteoblastic cells and deposited into the bone matrix (5). Osteopontin is highly phosphorylated and sulfated but is unlikely to be a primary nucleator of hydroxyapatite formation because it was found to appear before bone sialoprotein as a marker of an early stage of bone formation during the regenerative processes (10). Moreover, it has been demonstrated that osteopontin inhibits mineralization (3,10–13). Indeed, osteopontin is also expressed by a number of nonmineralizing tissues, as well as by transformed cells (14,15) and activated lymphocytes and macrophages (3,16), kidney epithelial cells, luminal epithelial cells of several organs (17) and smooth muscle cells in athelosclerotic lesions of the aorta (18). Osteocalcin is known to localize in bone, cementum and dentin, and in the formative cells of these hard tissues (5,18). Its function, however, is still not clear, although it has been implicated to play a role in delaying nucleation and in preventing excessive crystal growth (19). Bone sialoprotein appears to be unique to mineralized connective tissue. In addition, bone sialoprotein has the potential to mediate the initial formation of hydroxyapatite crystals (20–22).

It appears that Hertwig's epithelial root sheath cells are responsible for initiating the spatial and temporal differentiation of odontoblasts and cementoblasts into matrix-producing cells. The intermediate layer of cementum may be produced by Hertwig's epithelial root sheath cells (23–29). Moreover, Bosshardt *et al.* suggested that cementoblasts originate from Hertwig's epithelial root sheath (29,30), but species differences make the origin and differentiation of cementoblasts inconsistent between different studies, and therefore the understanding of cementogenesis is still incomplete. Mouri *et al.* demonstrated expression of alkaline phosphatase and osteopontin mRNA in cultured epithelial rests of Malassez cells from human periodontal ligament (31). Rincon *et al.* also demonstrated the production of osteopontin mRNA, but not of alkaline phosphatase mRNA, by cultured porcine epithelial rests of Malassez cells (32,33). Moreover, Hasegawa *et al.* reported that epithelial rests of Malassez cells are immunoreactive for osteopontin and ameloblastin during early cementum repair after the experimental induction of root resorption *in vivo* (26). It is conceivable that epithelial rests of Malassez cells, as well as Hertwig's epithelial root sheath cells, may have a direct or indirect role in the formation of cementum.

Some authors have suggested that epithelial rests of Malassez play a role in the maintenance of the periodontal space. Epithelial rests of Malassez cells were found to be stimulated to a more proliferating state as a result of orthodontic tooth movement, inflammatory conditions, or wound healing (34–39). A regrowth of epithelial rests of Malassez cells may be linked to reconstitution of the periodontal ligament and repair of the resorptive defect in the tooth surface. We demonstrated

previously that epithelial rests of Malassez cells have a high proliferation rate at the interface between epithelial rests of Malassez cells and human periodontal ligament fibroblasts and that the synthesis of type IV collagen and laminin is induced by direct interaction between epithelial rests of Malassez cells and human periodontal ligament fibroblasts (40). Considering the homeostasis of the periodontium, much seems to need to be done regarding the aspects of epithelial–mesenchymal interactions.

The present study was undertaken to determine whether epithelial rests of Malassez cells and human periodontal ligament fibroblasts at the interface *in vitro* have the ability to regulate alkaline phosphatase and the noncollagenous proteins, osteopontin, osteocalcin and bone sialoprotein, supporting their involvement in the maintenance of periodontal ligament.

Material and methods

Cell culture

Freshly extracted third molars from 24 patients between 17 and 25 years of age were obtained from the Oral Surgery Department, Tohoku University Graduate School of Dentistry. Informed consent was obtained from the patients prior to extractions. The human ethics board of Tohoku University Graduate School of Dentistry specifically granted permission for our project to work with human subjects. After washing the teeth several times with alpha minimal essential medium (COSMO BIO Co. Ltd, Tokyo, Japan), supplemented with 10% fetal bovine serum and antibiotics (60 μg/mL of kanamycin, 20 units/mL of penicillin G, 10 μg/mL of amphotericin B), human periodontal ligament explants attached to the mid-third of each root were removed carefully from the root with a scalpel. The explants were plated into 35-mm culture dishes using the supplemented alpha minimal essential medium and produced outgrowths that were primarily composed of fibroblasts. After 1 wk the explants were cultured in a modified serum-free medium [3 : 1 (v/v) MCDB153 medium; Sigma Chemical Co., St Louis, MO, USA] supplemented with 5 μg/mL of insulin (Sigma), 0.5 μg/mL of hydrocortisone (Sigma), 10 μg/mL of transferrine (Sigma), 14.1 μg/mL of phosphorylethanolamine (Sigma), 10 ng/mL of epidermal growth factor (Sigma): alpha minimal essential medium (COSMO BIO Co. Ltd) including 40 μg/mL of bovine pituitary extract (Kyokuto, Tokyo, Japan) and antibiotics (40). Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. This resulted in outgrowths of epithelial cells as well as of fibroblasts.

Differential adhesion to the surface in the presence of 0.075 mg/mL of protease solution (Sigma) 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)] from cultures that contained both cell types (41). Putative epithelial rests of Malassez cells cultured alone, and human periodontal ligament fibroblasts cultured alone, were used as controls.

Immunohistochemistry

The cells were fixed with 4% paraformaldehyde at room temperature for 10 min, and 3% H_2O_2 was used to inhibit endogenous peroxidase. The cells were then incubated with 5% normal goat serum for 30 min to block nonspecific binding, after which they were reacted at 4°C overnight with monoclonal mouse antihuman cytokeratin AE1/AE3 (1 : 50) (Dako, Carpinteria, CA, USA) to confirm the presence of epithelial cells. In addition, the cells were incubated at 4°C overnight with primary antibodies to monoclonal mouse antihuman osteopontin (0.1 μg/mL) (IBL, Gunma, Japan), polyclonal rabbit antihuman osteocalcin (1 : 250) (Biomedical Technology Inc., Stoughton, MA, USA) and polyclonal rabbit antihuman bone sialoprotein (LF-84) (1 : 400) (courtesy of Dr L. W. Fisher, National Institutes for Dental and Craniofacial Research, Bethesda, MD, USA) (42–45). After rinsing in phosphate-buffered saline, the cells were incubated with biotinylated immunoglobulin at room temperature for 30 min and stained by the avidin-biotinylated peroxidase complex using an ExtrAvidin® peroxidase staining kit (Sigma) and the 3-amino-9-ethylcarbazole chromogen kit (Sigma). Mayer's hematoxylin solution was applied for counterstaining. Phosphate-buffered saline, instead of the primary antibody, and rabbit serum and phosphate-buffered saline, instead of the primary antiserum, were used for control staining.

Proliferating, DNA-synthesizing cells in cocultures were identified by nuclear incorporation of 100 μmol/L of 5-bromo-2′-deoxyuridine for 24 h. Labeled nuclei were detected with monoclonal mouse antibody to 5-bromo-2′-deoxyuridine (Boehringer Mannheim Biochemica, Mannheim, Germany) for 30 min at 37°C. After washing three times in phosphate-buffered saline, the cells were incubated with horseradish peroxidase-conjugated goat antimouse immunoglobulin (Chemicon International Inc., Temecula, CA, USA) for 30 min at 37°C and stained with 3,3′-diaminobenzidine and H_2O_2.

Determination of alkaline phosphatase activity

The cells were fixed with 4% paraformaldehyde at room temperature for 10 min. To determine the localization of alkaline phosphatase in cultured cells, the cells were stained histochemically for alkaline phosphatase according to a modified method of the Azo-dye coupling method (46).

In situ hybridization

The oligonucleotide probes used for the *in situ* hybridization were synthesized by Nihon Gene Research Laboratories Inc. (Sendai, Japan). The sequences are shown in Table 1. A biotin label was added at the 3′ end. A computer-assisted search (GenBank) of the antisense sequences shown in Table 1, as well as that of sense sequences, revealed no significant homology with any known sequences other than those of the amelogenin chain, the alkaline phosphatase chain,

Table 1. Oligonucleotide probes used for the in situ hybridization

Oligo name	Sequence (5′–3′)	Mer	Label	Reference
Amelogenin	CAT GGG TTC GTA ACC ATA GGA AGG	24	3′ Biotin	(47)
Alkaline phosphatase	ACA TGA TGA CAT TCT TAG CCA CGT	24	3′ Biotin	(48)
Osteopontin	ATG GCT TTC GTT GGA CTT ACT TGG	24	3′ Biotin	(49)
Osteocalcin	GCG AGG AGT GTG TGA GGG CTC ATG	24	3′ Biotin	(50)
Bone sialoprotein	ATT TTT CAT TGA GAA AGC ACA GGC	24	3′ Biotin	(51)

the osteopontin chain, the osteocalcin chain and the bone sialoprotein chain.

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 phosphate-buffered saline containing RNase inhibitor at room temperature and 3% H_2O_2 was used to inhibit endogenous peroxidase. The specimens were hybridized with biotin-labeled probes, present in the hybridization solution, in the humid incubation chamber overnight at 37°C. After washing in phosphate-buffered saline, the specimens were reacted with blocking solution (5% bovine serum albumin, 500 µg/mL of normal sheep IgG, 100 µg/mL of salmon testicular DNA and 100 µg/mL of yeast tRNA in phosphate-buffered saline) at room temperature for 15 min. The cells were then incubated with ExtrAvidin® peroxidase solution (Sigma) at 37°C for 20 min and reacted with biotin-conjugated anti-avidin immunoglobulin (Sigma) in a humid chamber at 37°C for 30 min. After washing three times in phosphate-buffered saline, the sites of peroxidase activity were visualized using a solution containing 3,3′-diaminobenzidine and H_2O_2 and counterstained by Mayer's hematoxylin solution. Sense oligonucleotide probes were used for control staining.

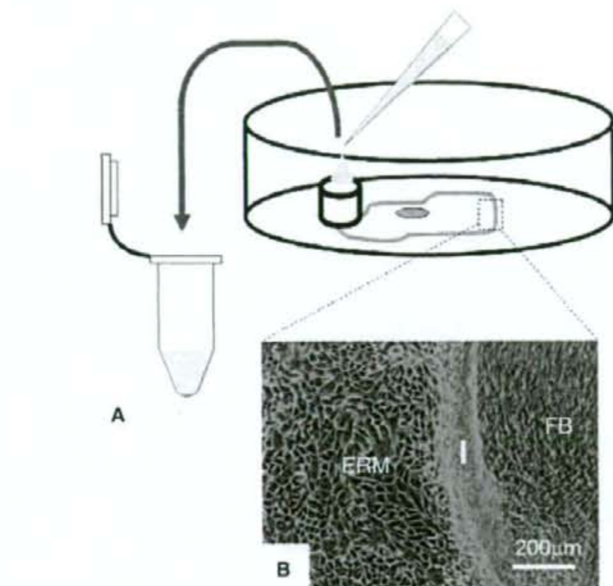

Fig. 1. (A) Schematic diagram showing the preparation of the samples at the interface between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts, except for only putative epithelial rests of Malassez cells or only human periodontal ligament fibroblasts away from the interface in the same dishes. The silicone cylinders (5 mm in diameter) pasted with white petrolatum on the bottom, were placed on the dishes. (B) Phase-contrast photomicrograph taken of primary cultured putative epithelial rests of Malassez cells (ERM) and of human periodontal ligament fibroblasts (FB) at the interface (I).

Reverse transcription polymerase chain reaction (RT-PCR)

The interface area cells, which are present between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts, were obtained using silicone cylinders (Fig. 1A,B) Briefly, silicone cylinders (5 mm in diameter) pasted with white petrolatum on the bottom, were placed on the dishes after aspirating the medium and washing twice with phosphate-buffered saline. Fifty microlitres of Buffer RLT (buffer containing guanidine thiocyanate and β-mercaptoethanol) (Qiagen Pty Ltd, Victoria, Australia) was added to the silicone cylinders. The cells disrupted with Buffer RLT were collected in the microcentrifuge tubes. Putative epithelial rests of Malassez cells cultured alone and human periodontal ligament fibroblasts cultured alone were sampled as controls. Total cellular RNA was isolated from cultured cells, according to the manufacturer's instructions, using an RNeasy® Mini Kit (Qiagen Pty Ltd), and 0.05 µg/µL of total RNA was used as a template for RT-PCR. One-step RT-PCR was performed using SuperScript™ one-step RT-PCR with the Platinum® Taq kit (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions and a Programmable Thermal Controller PTC-100 (MJ Research, Watertown, MA, USA). To quantify the expression of alkaline phosphatase and noncollagenous bone proteins (osteopontin, osteocalcin and bone sialoprotein), semiquantitative RT-PCR relative to glyceraldehyde-3-phosphate dehydrogenase was performed. Amplimers designed for alkaline phosphatase, osteopontin, osteocalcin,

Table 2. Primer pairs used for reverse transcription-polymerase chain reaction amplification

Gene (fragment)	Printer sequences	Denaturation/annealing/ extension (°C)	Cycle	Reference
Alkaline phosphatase (475 bp)	5'-ACGTGGCTAAGAATGTCATC-3' 5'-CTGGTAGGCGATGTCCTTA-3'	94/58/72	40	(52)
Osteopontin (126 bp)	5'CCAAGTAAGTCCAACGAAAG-3' 5'-GGTGATGTCCTCGTCTGTA-3'	94/58/72	40	(53)
Osteocalcin (230 bp)	5'-GGCAGCGAGGTAGTGAAGA-3' 5'-CTGGAGAGGAGCAGAACTG-3'	94/58/72	40	(54)
Bone sialoprotein (248 bp)	5'-CAACAGCACAGAGGCAGAA-3' 5'-CGTACTCCCCCTCGTATTC-3'	94/58/72	40	(51)
GAPDH (485 bp)	5'-TGTTTGTGATGGGTGTGAA-3' 5-ATGGGAGTTGCTGTTGAAG-3'	94/58/72	40	(55)

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

bone sialoprotein and glyceraldehyde-3-phosphate dehydrogenase, and related information, are provided in Table 2. The thermal profile for human alkaline phosphatase, osteopontin, osteocalcin, bone sialoprotein and glyceraldehyde-3-phosphate dehydrogenase amplification was 40 cycles, starting with denaturation for 1 min at 94°C followed by 1 min of annealing at 58°C and 1 min of extension at 72°C. The PCR products were subjected to electrophoresis, and digital images were obtained and analyzed with the use of IMAGE J (NIH image software, Bethesda, MD, USA). The data were consistent, as confirmed by three independent experiments. Statistical analysis of the results was carried out using the Bonferroni/Dunn post-hoc test, and p-values of < 0.05 were considered significant.

Results

Immunohistochemistry and determination of alkaline phosphatase activity

Putative epithelial rests of Malassez cells stained positive for broad-spectrum antibodies to cytokeratins (AE1/AE3), indicating their epithelial origin, whereas human periodontal ligament fibroblasts did not show cytokeratin expression at the interface in the same dishes (Fig. 2A). Putative epithelial rests of Malassez cells incorporated 5-bromo-2'-deoxyuridine more extensively than human periodontal ligament fibroblasts derived from the same periodontal ligament explant, indicating that putative epithelial rests of Malassez cells have a higher proliferation rate than human periodontal ligament fibroblasts (Fig. 2C).

Alkaline phosphatase activity was expressed strongly at the interface (Fig. 3A). Putative epithelial rests of Malassez cells cultured alone and human periodontal ligament fibroblasts cultured alone also stained strongly for alkaline phosphatase activity (Fig. 3B,C). Intense immunoreactivity for osteopontin was observed in both putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts at the interface (Fig. 3D). Osteopontin was also detected in putative epithelial rests of Malassez cells cultured alone and in human periodontal ligament fibroblasts cultured alone (Fig. 3E,F). Strong immunoreactivity for osteocalcin was observed in putative epithelial rests of Malassez cells but not in human periodontal ligament fibroblasts at the interface (Fig. 3G). In contrast, bone sialoprotein was detectable in human periodontal ligament fibroblasts but not in putative epithelial rests of Malassez cells at the interface (Fig. 3J). Osteocalcin and bone sialoprotein proteins were not detected in putative epithelial rests of Malassez cells cultured alone (Fig. 3H,K), but were present in human periodontal ligament fibroblasts cultured alone (Fig. 3I,L).

In situ hybridization to localize mRNAs for amelogenin, alkaline phosphatase and noncollagenous bone proteins

To localize the mRNA expression of amelogenin, alkaline phosphatase and noncollagenous bone proteins, the cells were hybridized *in situ* with biotin-labeled antisense oligo-DNA probes. Putative epithelial rests of Malassez cells showed strongly positive signals for amelogenin mRNA. However, the amelogenin mRNA signal was not detectable in human periodontal ligament fibroblasts (Fig. 2B). These results supported that putative epithelial rests of Malassez cells are different from human periodontal ligament fibroblasts and are derived from the odontogenic epithelial origin.

In situ hybridization analyses also showed considerable differences between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts and reflected the histochemical and immunohistochemical data. The mRNAs of alkaline phosphatase, osteopontin and osteocalcin were expressed strongly in putative epithelial rests of Malassez cells at the interface, whereas bone sialoprotein mRNA expression was not detectable (Fig. 4A,D,G,J). On the other hand, mRNAs of alkaline phosphatase, osteopontin and bone sialoprotein were expressed strong in human periodontal ligament fibroblasts at the interface, whereas osteocalcin mRNA expression was not detectable (Fig. 4A,D,G,J). Putative epithelial rests of Malassez cells cultured alone showed strong positive signals for alkaline phosphatase and osteopontin mRNAs (Fig. 4B,E). mRNA for osteocalcin was detectable only very weakly (Fig. 4H) and mRNA for bone sialoprotein was not expressed at all in putative

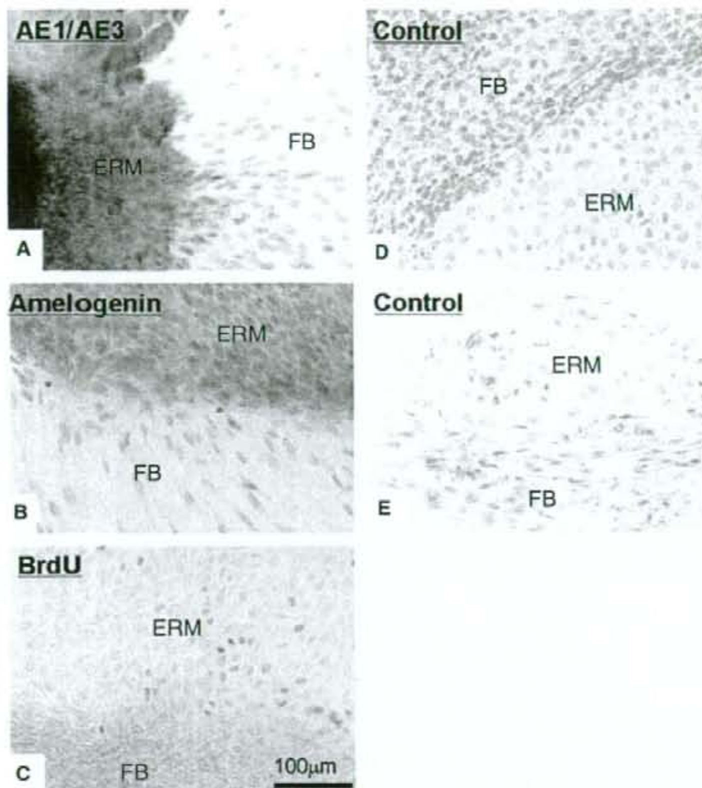

Fig. 2. (A) Photomicrograph showing strong immunoreactivity for cytokeratin AE1/AE3 in putative epithelial rests of Malassez cells. Immunostaining for cytokeratin in human periodontal ligament fibroblasts is not present. (B) Photomicrograph showing strong immunoreactivity for amelogenin mRNA in putative epithelial rests of Malassez cells. Immunostaining for amelogenin mRNA in human periodontal ligament fibroblasts is not present. (C) Photomicrograph showing assessment of proliferation by 5-bromo-2'-deoxyuridine labeling in putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts. (D) Photomicrograph showing negative-control immunostaining in putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts. (E) Photomicrograph showing the negative control hybridized *in situ* with biotin-labeled sense oligo-DNA probes for amelogenin mRNA in putative epithelial rests of Malassez cells and in human periodontal ligament fibroblasts. ERM, epithelial rests of Malassez cells; FB, human periodontal ligament fibroblasts.

epithelial rests of Malassez cells cultured alone (Fig. 4K). On the other hand, mRNAs for osteopontin, osteocalcin and bone sialoprotein were detectable in human periodontal ligament fibroblasts cultured alone (Fig. 4F,I,L), and a strong signal for alkaline phosphatase mRNA was detected in human periodontal ligament fibroblasts cultured alone (Fig. 4C).

RT-PCR

The expression of four genes (mRNAs of alkaline phosphatase, osteopontin, osteocalcin and bone sialoprotein) was investigated in three cell populations, both putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts at the interface, putative epithelial rests of Malassez cells cultured alone and human periodontal ligament fibroblasts cultured alone, using RT-PCR (Fig. 5). The mRNA intensities relative to that of glyceraldehyde-3-phosphate dehydrogenase are presented in Fig. 6. The expression of mRNAs for alkaline phosphatase and bone sialoprotein were significantly stronger at the interface between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts ($p < 0.05$). On the other hand, there were no significant differences in the relative intensities of mRNAs for osteopontin and osteocalcin among the three cell populations. The mRNA for osteocalcin was detectable only very weakly, and the mRNA for bone sialoprotein was not expressed at all in putative epithelial rests of Malassez cells cultured alone. A strong signal for alkaline phosphatase mRNA expression was detected in human periodontal ligament fibroblasts cultured alone, and mRNAs for osteopontin, osteocalcin and bone sialoprotein were detectable in human periodontal ligament fibroblasts cultured alone.

Discussion

Amelogenin is the major enamel protein produced by ameloblasts at the differentiation and secretory stages (3). Amelogenin has been observed to be localized in the enamel matrix, in normal ameloblasts, in odontogenic tumor cells, in Hertwig's epithelial root sheath cells and their extracellular matrices *in vivo*, in mantle dentin and in odontoblasts (3,30,56–60). In the present study, amelogenin mRNA expression distinguished putative epithelial rests of Malassez cells from human periodontal ligament fibroblasts at the interface, and putative epithelial rests of Malassez cells were the odontogenic epithelial cells derived from Hertwig's epithelial root sheath (Fig. 2B). Bosshardt *et al.* suggest that Hertwig's epithelial root sheath cells occasionally assume a lingering ameloblastic activity at the start of root formation in the pig, but they do not support the hypothesis of a causal relationship between enamel matrix proteins and cementogenesis (30). Moreover, they indicated that ectopic enamel deposits