5）口腔機能障害への対応

正常な咀嚼嚥下機能の発現には，粘膜の保湿が不可欠であるが，口腔乾燥患者では，唾液による粘膜の保湿が不十分となり，スムーズな動きが制限され，水分摂取時にむせたり誤嚥する場合が多くなる．口腔乾燥に関連した咀嚼嚥下障害が認められる場合には，積極的な保湿を目的とした口腔ケアが有用となる．この場合，洗口液（絹水®やオーラルウェット®）などの湿潤剤配合洗口液を用いるが，要介護高齢者などに対しては，洗口法よりも，スポンジブラシを用いた粘膜への塗布やスプレー容器による噴霧が効果的である．オーラルバランス®も粘膜への停留効果により効果がある．

口腔乾燥がある場合には，食前の口腔ケアが必須で，食べる前に粘膜の保湿を目的としたケアを行うと臨床的効果が高い．また，舌を前後左右に動かしてもらう舌体操も効果的である．指による唾液腺へのマッサージ効果も有効である[8]．

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7. 食事指導・生活指導

唾液低下や口腔乾燥があると，正常な口腔機能を発揮できなくなることで，咀嚼障害や嚥下障害，味覚低下などの症状が発現しやすい．これは，唾液が粘膜保湿や口腔の自浄作用などに役立っており，口腔内の潤滑剤の役目も併せもつことによる．唾液が減少すると，粘膜の再生力や抵抗力が低下して，粘膜が傷つきやすくなったり，感覚低下を起こすことも考えられる．

重度の口腔乾燥では，経口摂取が困難になり，低栄養や脱水症状に陥る可能性がある．誤嚥防止や栄養補給を目的として，安易に経管栄養に移行してしまうと，口腔への適度な刺激がなくなるために，さらに唾液が減少して，咀嚼や嚥下の機能低下が進んでしまう．したがって，口腔乾燥や唾液分泌低下がある患者では，正常な口腔機能が発揮できるように，口腔機能の維持改善，食前の口腔粘膜保湿と嚥下しやすい食事形態などについて考慮することが必要となる[1)]．

1 食事機能の維持・改善

口腔乾燥がある患者では，より口腔機能を発揮できるような配慮を行う．すなわち，乾燥症状に対する口腔粘膜保湿，唾液性状の改善と唾液による嚥下訓練，義歯の調整改善などである．

粘膜保湿は，水分の補給のみでは，唾液分泌につながらない場合も多いので，粘膜への直接的な保湿を行う．乾燥した口腔粘膜では，水分が粘膜表面を流れるだけで，十分な保湿はむずかしい場合が多いので，絹水®やオーラルウェット®などの保湿剤や蒸散防止効果のあるオーラルバランス®などの保湿ジェルを応用するとよい．空嚥下は，安静時の唾液嚥下であるが，唾液分泌が低下すると，空嚥下の回数が極端に減少してしまう．通常は，約3分に1回の空嚥下であるのに対して，唾液が少なくなると，何時間も空嚥下ができない状況になる場合もある．空嚥下ができないと，嚥下のウォーミングアップができないため，むせや誤嚥が生じやすくなる．麻痺がないにもかかわらず嚥下障害をきたしやすいのは，このためでもある．さらに，ある程度の唾液量が存在しても，粘性が亢進した唾液が，常に粘膜をおおってしまうと，感覚低下をきたし，その結果として，咳反射や嚥下反射が低下することになる．粘性亢進した唾液の除去や清掃，唾液性状の改善も必要となる．

このような場合には，臨床上，唾液の嚥下訓練が効果的である．唾液が少ない場合には，人工唾液として，保湿剤や保湿ジェルなどを応用する．水分のみでは，咽喉頭部へ流入しやすいので，注意する．

口腔乾燥や唾液低下の状況下では，義歯の調整や管理も重要である．唾液が豊富な状態では，何ともなかった義歯が，唾液低下のために，急に合わなくなることが多い．唾液は，

粘膜と義歯の間で生じる物理的な維持に役立っている．唾液が粘膜を適度な保湿状態に保つことで，適度な軟らかさや滑らかさによる安定にも役立っている．したがって，唾液が少なくなると，義歯の安定が悪くなり，粘膜に傷がつきやすくなったり，義歯が脱落しやすくなる．唾液低下や口腔乾燥で義歯不安定になった場合には，義歯内面や顎堤に保湿剤や保湿ジェルを塗布すると安定性が増すので，唾液の評価を忘れないようにする．義歯のトラブルがある場合には，他の原因も関連していることがあるので，歯科医師の診察を受けることも必要である．

食事指導

唾液低下や口腔乾燥患者では，唾液の評価，特に唾液量，唾液分布の状態，口腔粘膜の乾燥度，空嚥下の状態，むせの状態などを評価，観察する．

口腔乾燥による咀嚼嚥下困難に対しては，食前にうがいや口腔ケアなどにより口腔粘膜や舌を潤すだけでも効果がある場合も多い．また，口腔の状況に合った食事内容や食物形態を採用することが望ましい[1,2]（表24）．

●表24　口腔乾燥時に食べやすい食形態

1	適度な粘度があり，食塊形成がしやすいもの
2	口腔内や咽頭を，滑らかに通過しやすいもの
3	べたつかず，のどごしがいいもの
4	密度が一定のもの（水分と固形物がバラバラにならないもの）

（文献1より改変）

口腔が乾燥しやすい場合には，食物を口腔内で処理しにくく，誤嚥しやすくなっているので，食べやすく飲み込みやすい調理形態や適切な栄養がとれる食材を採用する．汁にトロミをつけたり，軟らかくなるまで，煮込む，ゼラチンを利用する，葛粉，片栗粉でトロミをつけた食品を採用する，などといった工夫をする（表25）．

口腔乾燥による重度な嚥下障害があると，家族と同じ食材をミキサーにかけて，トロミをつけたりする．ミキサー食やキザミ食だけでは，かえって誤嚥しやすくなるので，トロミをつけることを忘れないようにする．

また，食器や料理の形を工夫したり，食材の色などにアクセントを加える，季節感のある食材や食器などの利用，好きなものの調理などで，食欲をそそるようにする．

口腔乾燥や唾液低下で，注意したい食材としては，液体や，硬くて食べにくいもの，食品内の水分が少ないもの，繊維の多いもの，練り製品や魚貝類，口腔内に付着しやすいもの，酸味が強すぎるものなどで，口腔症状に応じて配慮する（表26）．

水分やお茶，味噌汁などの液体は，乾燥した粘膜をそのまま流れていきやすいので，1回量を少なくするかトロミをつける．リンゴや梨，焼いた肉，干し物，餅などは調理後に包丁を入れるなどして食べやすくする．また，繊維の多いものや練り製品なども包丁を入れる．義歯患者では，特に配慮が必要である．

食品に水分が少ない食材は粘膜の水分を吸収してしまうので，嚥下しにくくなる．あらか

●表25　食材の工夫

	トロミをつける
1	完成した食材に葛粉や片栗粉でトロミをつける． トロメリン®やトロミアップ®などの増粘剤を利用する．
2	軟らかくなるまで煮込む
	ゼラチンを利用する
3	タンパク質食品はゼリーで固め，テリーヌやプリン，ムースにする． フルーツや野菜などはミキサーにかけてからゼリーなどにする．
4	トロミをつけた食品の採用 葛あんかけや牛乳羹などをメニューに加える．
	その他
5	卵は卵どうふや茶碗蒸しなどの蒸した料理がよい． 山芋とろろ，マグロのとろろかけなどは食べやすい． イモ類はつぶして裏ごしをして，適度な水分を加えて調節する．

●表26　注意したい食品

1	液　体
2	固くて食べにくいもの
3	食品内に水分が少ないもの
4	繊維の多いもの
5	練り製品や魚介類
6	口腔内に付着しやすいもの
7	酸味が強すぎるもの

じめ口腔内の保湿をしてから食べるか嚥下しやすいように1回量を小さくしておく．ワカメや海苔などの海藻類，青菜などは口腔粘膜に付着しやすいので，汁物の具としては避ける．

柑橘類や酢物，梅干，レモンジュースなど酸味のある食品などが，唾液分泌改善の目的で使用されることが多いが，唾液分泌量そのものが少ない患者では，口腔内の自浄作用や唾液の緩衝作用が低下しているので，かえって，う蝕の増加やカンジダ症の発症などを促したり，酸蝕症の症状をきたすことがある．また，口腔乾燥で傷ついた舌粘膜や口腔粘膜の疼痛をきたすことがあるので，注意が必要である．

嚥下訓練と食事形態

口腔乾燥が重度で，嚥下困難を生じている場合は，嚥下訓練が必要となる．最も安全で効果的な嚥下訓練は唾液の嚥下訓練である．嚥下するだけの唾液がない場合には人工唾液や保湿剤を使用する．水分だと口腔粘膜上を流れてしまうので，唾液が少ない場合には，唾液の流れと比較的近く，粘膜への親和性がよい絹水®やオーラルウェット®といった保湿剤などを利用する．

嚥下訓練を開始する場合には，ゼリー食が効果的であるとされる．ゼラチンはゾル（溶解）ゲル（固体）転位を有し，口腔内の温度で表面が溶け，しかも内面は固まったままの状態を保つため，食感が滑らかで食塊形成が容易にできる．ゼラチンとよく似た食材に寒天があるが，口腔内でバラバラになりやすいので，嚥下困難には不向きである．増粘剤による食塊形成は，熱いときは流れがよいが，食材が冷えると吸着性や粘度が増すので，不適当である．食事形態は口腔状態や嚥下機能に合わせて，対応していく[3~5]（表27）．

●表27　嚥下訓練時の食形態

嚥下食Ⅰ	スープ，ジュースなどにゼラチンで固めたもの
嚥下食Ⅱ	野菜，魚の煮物をミキサーにかけ，ゼラチンで固めたもの
嚥下食Ⅲ	ピューレ状のもの，ムース，増粘剤やでんぷんなどで，トロミをつけたもの
移行食	軟らかく煮込んでいるもの，比較的水分の多い食品を刻んだもの，または一口大にしたもの

（文献1より改変）

4 生活指導

口腔乾燥のある患者では，口腔乾燥の改善でQOLが向上できることを説明することと，口腔ケアのプラン作成や生活指導が重要となる[6]（表28）．

口腔乾燥の原因に対しては，唾液分泌との関連性や症状との関係について説明をし，理解を求める．原因が薬剤にある場合には，主治医に相談して，原因薬剤の変更や減量を検討をお願いする．一般に，短期間では副作用の発現しない薬剤でも長期服用すると徐々に副作用発現してくるものが多いので，注意する．特に，口腔内の違和感や不快感，べたつき感を解消する目的で精神安定剤などを服用していると，唾液分泌低下を助長したり，薬効が切れたときの違和感が増すために，症状が複雑になりやすい．

口呼吸や夜間のいびきなどがある場合には，睡眠時無呼吸症候群やいびきに対応できる内科的治療や，歯科スプリントなどの口腔装置の作製も効果的である．鼻疾患による口呼吸がある場合には，鼻疾患の治療を行う．口腔機能低下による唾液腺刺激低下が原因の場合には，義歯治療や咬合治療，唾液腺マッサージなどを行う．また，食事訓練も有効となる．シェーグレン症候群などの全身疾患の場合は，治療を行う．

●表28　口腔乾燥患者の口腔ケアプラン

	口腔乾燥の原因に対するアプローチ
1	薬剤，口呼吸やいびき，鼻疾患による口呼吸 口腔機能低下による唾液腺刺激低下
2	口腔粘膜の保湿
3	水分摂取のコントロール
4	嗜好品への指導
5	夜間排尿への配慮
6	その他

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慢性の口腔乾燥に対して，水分を多く飲むことで対応している場合には，浸透圧調整能力が低下していることも多く，体への水分補給につながらず排尿頻度の増加になっている場合が多い．そのために夜間排尿の頻度が増えて，睡眠障害をきたしている場合は，漢方製剤などを用いて体質改善することも考慮すべきである．

寝たきり患者などで，口腔乾燥がみられる場合には，単なる清掃としての口腔ケアではなく，保湿ケアを同時に行うことが重要となる．

唾液分泌低下があると，咽喉頭部の乾燥感も自覚するようになるため，のど飴を常用する患者が多くなる．健康食品的なイメージで多用すると，糖分を多く含んでいるものや酸性のものもあるので，細菌叢の変化をきたしたり，う蝕の発症要因になることも多い．口腔乾燥のために，のど飴を常用している場合には，原因となる口腔乾燥や唾液分泌への対応が必要であり，のど飴の常用はできるだけ避けるように指導する．どうしても，のど飴が必要な場合には，ノンシュガーのものにする．

唾液分泌低下や口腔乾燥で，舌苔が増加することがある．舌苔は，上部消化管や全身状態の結果として生じており，無理に除去すると，舌粘膜に傷がつく可能性もあるので，軽い力で，清掃するだけでよい．パイナップルを用いて舌苔除去を行う場合もあるが，タンパク質分解酵素によって舌粘膜上の糸状乳頭が溶解している状態であり，口腔ケアとしては使用しないようにする[7]．

唾液分泌低下や口腔乾燥で，食事の変化や嚥下障害を自覚した場合には，より専門的に対応することで，よりいっそうのQOL向上が図れる．

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Mechanisms Involved in Enhancement of Osteoclast Formation and Function by Low Molecular Weight Hyaluronic Acid*

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Hyaluronic acid (HA) is a component of the extracellular matrix that has been shown to play an important role in bone formation, resorption, and mineralization both *in vivo* and *in vitro*. We examined the effects of HA at several molecular weights on osteoclast formation and function induced by RANKL (receptor activator of NF-κB ligand) in a mouse monocyte cell line (RAW 264.7). HA at M_r < 8,000 (low molecular weight HA (LMW-HA)) enhanced tartrate-resistant acid phosphatase-positive multinucleated cell formation and tartrate-resistant acid phosphatase activity induced by RANKL in a dose-dependent manner, whereas HA at M_r > 900,000 (high molecular weight HA (HMW-HA)) showed no effect on osteoclast differentiation. LMW-HA enhanced pit formation induced by RAW 264.7 cells, whereas HMW-HA did not, and LMW-HA stimulated the expression of RANK (receptor activator of NF-κB) protein in RAW 264.7 cells. In addition, we found that LMW-HA enhanced the levels of c-Src protein and phosphorylation of ERKs and p38 MAPK in RAW 264.7 cells stimulated with RANKL, whereas the p38 MAPK inhibitor SB203580 inhibited RANKL-induced osteoclast differentiation. This enhancement of c-Src and RANK proteins induced by LMW-HA was inhibited by CD44 function-blocking monoclonal antibody. These results indicate that LMW-HA plays an important role in osteoclast differentiation and function through the interaction of RANKL and RANK.

Hyaluronan, or hyaluronic acid (HA),[1] is a long polysaccharide chain that is made of repeating disaccharide units of *N*-acetylglucosamine and glucuronic acid. HA is the most abundant glycosaminoglycan in mammalian tissue and is present in high concentrations in connective tissues as well as in skin, the vitreous humor of the eye, cartilage, and umbilical cord tissue. The largest single reservoir is the synovial fluid of diarthroses joints, where concentrations of 0.5–4 mg/ml have bee found (1, 2). In rheumatoid arthritis and osteoarthritis, the molecular weight and concentration of HA in synovial fluid are reduced, probably due to abnormal biosynthesis by synovial type B cells and free radical depolymerization of the HA chain (3). These changes in the rheological properties of synovial fluid in arthritic joints may contribute to disease progression because articular cartilage, subchondral bone, and synovial connective tissues are subjected to increased mechanical stress when the viscoelastic and lubricating properties of HA are diminished.

Many studies have reported the biological effects of HA on chondrocytes (4–11). Furthermore, HA affects the catabolic activity of the cartilage matrix by inducing the production of TIMP-1 (tissue inhibitor of metalloproteinase-1) by bovine chondrocytes (6). Sakamoto *et al.* (12) reported that high molecular weight HA (HMW-HA) injected into the joint cavity becomes attached to the surface of articular cartilage, where it exerts chondroprotective effects. Extrinsic HA has also been shown to penetrate degenerated cartilage and to reach the surface of chondrocytes (13). In addition, *in vitro* studies using synovial fibroblasts have indicated that synovial type B cells, the major source of synovial HMW-HA, lead to *de novo* synthesis of HMW-HA (14).

In a previous study utilizing high performance liquid chromatography (15), we demonstrated that the molecular weights of HA in synovial fluid from temporomandibular joint samples from patients with internal derangement and osteoarthritis are decreased probably due to free radical depolymerization of the HA chain and/or abnormal biosynthesis by the synovium. The results of that study shed light on the mechanisms of the changes in joint lubrication and the pathology of the temporomandibular joint. Several researchers have reported the involvement of the interaction between CD44 and HA in bone resorption (2, 16, 17). However, less attention has been paid to the effect of low molecular weight HA (LMW-HA) on osteoclast formation induced by RANKL (receptor activator of NF-κB ligand). In this study, we examined the effects of HA at several molecular weights on osteoclastogenesis induced by RANKL and found that LMW-HA enhanced both osteoclast formation and function *in vitro*.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—HA at several molecular weights (4-mer, 12-mer, and M_r = 8,000, 900,000, and 2,000,000) was kindly supplied by Seikagaku Corp. (Tokyo, Japan). Human recombinant soluble RANKL was purchased from Pepro Tech EC Ltd. (London, United Kingdom). Anti-c-Src monoclonal antibody and anti-RANK polyclonal antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-p38 MAPK, anti-phospho-p38 MAPK, anti-ERK, and anti-phospho-ERK polyclonal antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA). SB203580 was purchased from Calbiochem. CD44 function-blocking monoclonal antibody (BRIC 235) was obtained from the International Blood Group Reference Laboratory (Bristol, United Kingdom).

Cell Culture—Mouse monocyte RAW 264.7 cells were maintained in α-minimal essential medium (Invitrogen) supplemented with 10% fetal

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[1] The abbreviations used are: HA, hyaluronic acid; HMW-HA, high molecular weight hyaluronic acid; LMW-HA, low molecular weight hyaluronic acid; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; TRAP, tartrate-resistant acid phosphatase; OCL, osteoclast-like cell; JNK, c-Jun N-terminal kinase.

bovine serum (Invitrogen), 100 units/ml penicillin G, and 100 μg/ml streptomycin in collagen-coated dishes (Asahi Techno Glass, Chiba, Japan).

Cell Viability—RAW 264.7 cells were plated in 96-well plates at a concentration of 5×10^2 cells/well 1 day before the experiment and then stimulated with RANKL and HA at several molecular weights. The stimulated cells were cultured for 1, 2, 5, or 6 days, after which a stock 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (2.5 mg/ml, 20 μl/well; Sigma) was added to the wells, and the plates were incubated for 4 h. Next, acid/isopropyl alcohol (100 μl of 0.04 N HCl in isopropyl alcohol) was added and mixed thoroughly, and the plates were read using a Multiskan Bichromatic microplate reader (Labsystems, Helsinki, Finland), with a test wavelength of 540 nm and a reference wavelength of 620 nm (18).

Differentiation of Osteoclasts—Osteoclasts were detected by staining with tartrate-resistant acid phosphatase (TRAP) (Sigma) (19). In brief, RAW 264.7 cells were cultured in 96-well plates (5.0×10^2 cells/well) in the presence of RANKL (40 ng/ml), HA at several molecular weights, or SB203580. After culturing for the indicated times, the adherent cells were fixed and stained for TRAP activity. TRAP-positive multinucleated cells containing three or more nuclei were considered to be osteoclast-like cells (OCLs) and were counted under a microscope.

TRAP Activity—For the TRAP activity assay, RAW 264.7 cells were cultured in 96-well plates (5.0×10^2 cells/well) in the presence of RANKL (40 ng/ml), HA (100 ng/ml) at several molecular weights, or SB203580 for 6 days. The treated RAW 264.7 cells were suspended in 25 μl of phosphate-buffered saline, pH 7.2, and then frozen and thawed three times. TRAP activities in the supernatants were analyzed using a phenyl phosphate substrate kit (Sanseiphospha KII-Test-Wako, Wako, Osaka, Japan) according to the manufacturer's instructions (20).

Bone Resorption Assay—To estimate bone resorption activity, RAW 264.7 cells were cultured for 14 days with RANKL (40 ng/ml) and HA (100 ng/ml) at several molecular weights on BD BioCoat™ Osteologic™ multitest slides, which consisted of submicron synthetic calcium phosphate thin films coated onto various culture vessels (BD Biosciences). The cells were removed using 6% NaOCl and 5.2% NaCl, and the number of the resorption pits formed in each well was counted under a microscope.

Western Blot Analysis—RAW 264.7 cells (1×10^6) were cultured in α-minimal essential medium containing 10% fetal calf serum in the presence of RANKL (40 ng/ml) and HA (100 μg/ml) at several molecular weights (M_r = 8,000 and 2,000,000), SB203580 (10^{-6} M), or BRIC 235 (5 μg/ml) on 6-well plates. Adherent cells were washed twice with phosphate-buffered saline and lysed in cell lysis buffer (75 mM Tris-HCl containing 2% SDS and 10% glycerol, pH 6.8). Protein contents were measured using a *DC* protein assay kit (Bio-Rad). The samples subjected to 10% SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). Nonspecific binding sites were blocked by immersing the membranes in 10% skim milk in phosphate-buffered saline for 1 h at room temperature, and the membrane was washed four times with phosphate-buffered saline, followed by incubation with diluted primary antibody for 2 h at room temperature. Anti-c-Src, anti-ERK, anti-phospho-ERK, anti-p38 MAPK, anti-phospho-p38 MAPK, and anti-RANK primary antibodies and horseradish peroxidase-conjugated anti-mouse, anti-rabbit, and anti-goat IgG secondary antibodies (Santa Cruz Biotechnology, Inc.) were used in this experiment. After washing the membranes, chemiluminescence was produced using ECL reagent (Amersham Biosciences) and detected with Hyperfilm-ECL (Amersham Biosciences). After exposure to film, the membranes were stained with Coomassie Brilliant Blue G-250 to confirm equal loading.

Statistical Analysis—Statistical differences were determined using an unpaired Student's *t* test with Bonferroni's correction for multiple comparisons. All data are expressed as the mean ± S.E.

RESULTS

Effects of RANKL and HA on Cell Growth—We examined the effects of RANKL and HA at several molecular weights on the proliferation of RAW 264.7 cells using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide viability assay. HA had no effect on RAW 264.7 cell growth after 1, 2, 5, and 6 days of culture with RANKL (data not shown).

LMW-HA Up-regulates Osteoclast Differentiation Induced by RANKL—Culturing LMW-HA activated the differentiation of RAW 264.7 cells into OCLs via RANKL in a dose-dependent manner (Fig. 1, *A* and *B*), whereas HMW-HA had almost no effect. The number of TRAP-positive multinucleated cells was maximized at higher concentrations (100 ng/ml) in this experiment; however, LMW-HA alone did not induce OCL formation (data not shown). Incubation with LMW-HA and RANKL for 0–2 days was adequate to enhance OCL formation in RAW 264.7 cells (Fig. 1*C*).

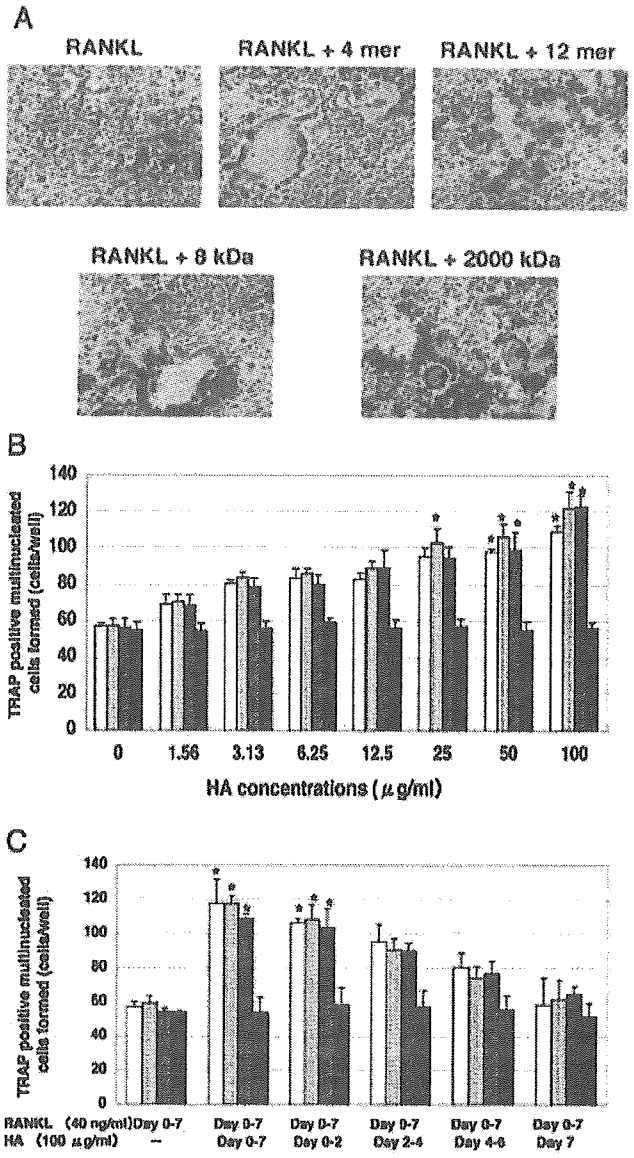

FIG. 1. **Effect of HA on osteoclast differentiation induced by RANKL.** RAW 264.7 cells were stimulated with RANKL (40 ng/ml) and HA at several molecular weights for 6 days. *A*, the images show OCL formation. *B*, the number of OCLs was counted after the cells were stained for TRAP activity. Data are expressed as the mean ± S.D. of triplicate cultures. The experiment was performed three times, with similar results obtained in each experiment. *, $p < 0.05$ (Student's *t* test). *C*, shown are the results from a time course experiment. □, 4-mer; ▨, 12-mer; ▨, M_r = 8,000; ■, M_r = 2,000,000.

LMW-HA Up-regulates TRAP Activity Induced by RANKL—We examined the effects of RANKL in the presence of HA at several molecular weights on the TRAP activity of RAW 264.7 cells using the phenyl phosphate substrate method. As shown in Fig. 2, RANKL alone enhanced TRAP activity in RAW 264.7 cells; however, when the cells were incubated with both RANKL and LMW-HA, the level of TRAP expression was 1.5-fold higher than in cells treated with RANKL alone. In contrast, HMW-HA had no effect on RANKL-induced TRAP activity in RAW 264.7 cells.

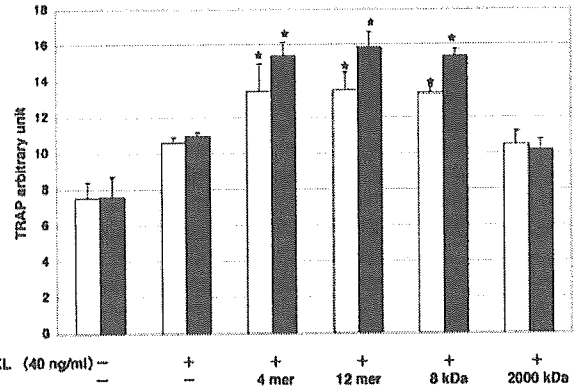

FIG. 2. **Effects of HA on TRAP activity induced by RANKL.** RAW 264.7 cells were stimulated with RANKL (40 ng/ml) in the presence of HA (100 μg/ml) at several molecular weights for 6 days. TRAP activity was determined as described under "Experimental Procedures." Data are expressed as the mean ± S.D. of triplicate cultures. The experiment was performed three times, with similar results obtained in each experiment. *, $p < 0.05$ (Student's t test). □, 10 μg/ml. ■, 100 μg/ml.

LMW-HA Stimulates Bone Resorption Induced by RANKL—To determine whether LMW-HA affects osteoclast function, differentiated RAW 264.7 cells were cultured on Osteologic™ multitest slides with RANKL (40 ng/ml) in the presence of HA (100 μg/ml) at several molecular weights. LMW-HA enhanced the stimulatory effect of RANKL on bone resorption (Fig. 3), whereas it had no effect on basal bone resorption, with or without RANKL.

LMW-HA Enhances the Expression of RANK Protein in RAW 264.7 Cells—We also examined RANK expression in RAW 264.7 cells by immunoblot analysis. RAW 264.7 cells were cultured for 6–72 h in the presence or absence of RANKL and HA at several molecular weights. Interestingly, treatment with LMW-HA alone induced RANK expression and enhanced the expression level in RAW 264.7 cells treated with RANKL, whereas HMW-HA had no effect on the level of RANK protein expression (Fig. 4).

LMW-HA Enhances RANKL-induced Expression of c-Src Protein in RAW 264.7 Cells—Next, we investigated the effect of RANKL and HA at several molecular weights on the expression of c-Src protein in RAW 264.7 cells by Western blotting. As shown in Fig. 5, the level of c-Src protein expression was increased in cells following stimulation with RANKL. Furthermore, c-Src protein expression was up-regulated upon the addition of LMW-HA in a time-dependent manner up to 72 h.

LMW-HA Enhances RANKL-induced Activation of MAPKs in RAW 264.7 Cells—The effects of RANKL and HA at several molecular weights on the activation of ERKs at M_r = 42,000 and 44,000 in RAW 264.7 cells were investigated (Fig. 6). Western blot analysis revealed that phosphorylated ERKs were detected within 15 min and reached a plateau at 30 min after the addition of RANKL. When the cells were incubated with both RANKL and LMW-HA, the level of ERK phosphorylation was higher than in cells treated with RANKL alone. In contrast, the total amounts of ERK protein were not affected by treatment with RANKL and HA.

We also examined the effects of RANKL and HA at several molecular weights on the phosphorylation of p38 MAPK in osteoclast precursors. Fig. 7 shows the time course of changes in the level of p38 MAPK phosphorylation in response to RANKL in RAW 264.7 cells. p38 MAPK was phosphorylated within 30 min in response to RANKL, and phosphorylation reached a maximum level within 60 min. LMW-HA stimulated the RANKL-induced phosphorylation of p38 MAPK; however,

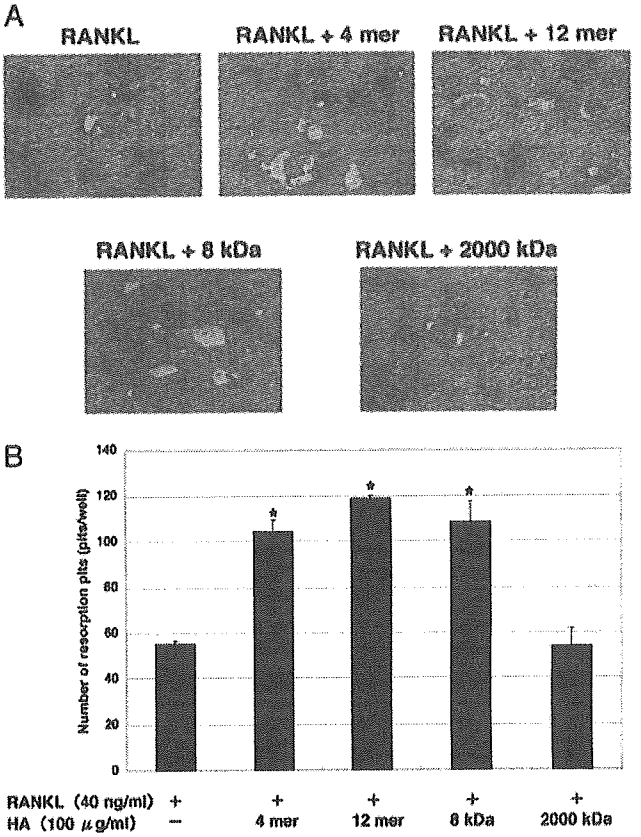

FIG. 3. **Effect of HA on bone resorption induced by RANKL.** RAW 264.7 cells were cultured with RANKL (40 ng/ml) in the presence of HA (100 μg/ml) at several molecular weights on Osteologic™ plates for 14 days. *A*, the images show pit formation. *B*, the number of resorption pits was counted after removing the cells. Data are expressed as the mean ± S.D. of triplicate cultures. The experiment was performed three times, with similar results obtained in each experiment. *, $p < 0.05$ (Student's t test).

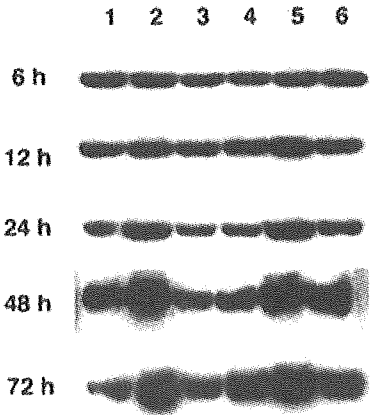

FIG. 4. **Detection of RANK in RAW 264.7 cells treated with HA.** RAW 264.7 cells were exposed to RANKL (40 ng/ml) in the presence of HA (100 μg/ml) at several molecular weights for the times indicated, and whole lysates were subjected to Western blot analysis. *Lane 1*, control; *lane 2*, LMW-HA (M_r = 8,000); *lane 3*, HMW-HA (M_r = 2,000,000); *lane 4*, RANKL (40 ng/ml); *lane 5*, RANKL (40 ng/ml) and LMW-HA (M_r = 8,000); *lane 6*, RANKL (40 ng/ml) and HMW-HA (M_r = 2,000,000).

there was no change in the level of p38 MAPK phosphorylation upon treatment with LMW-HA alone. Furthermore, the total amounts of p38 MAPK protein in RAW 264.7 cells stimulated

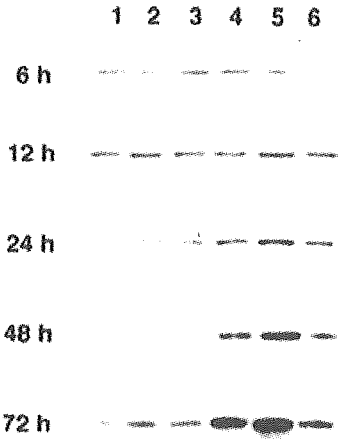

FIG. 5. **Detection of c-Src expression in RAW 264.7 cells treated with HA.** RAW 264.7 cells were exposed to RANKL (100 ng/ml) in the presence of HA (100 μg/ml) at several molecular weights, and whole lysates were subjected to Western blot analysis. *Lane 1*, control; *lane 2*, LMW-HA (M_r = 8,000); *lane 3*, HMW-HA (M_r = 2,000,000); *lane 4*, RANKL (40 ng/ml); *lane 5*, RANKL (40 ng/ml) and LMW-HA (M_r = 8,000); *lane 6*, RANKL (40 ng/ml) and HMW-HA (M_r = 2,000,000).

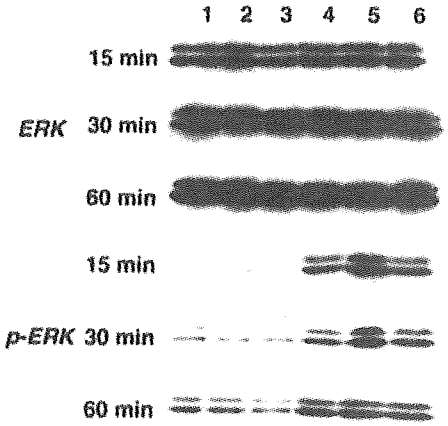

FIG. 6. **Detection of phosphorylated ERK protein in RAW 264.7 cells treated with HA.** RAW 264.7 cells were exposed to RANKL (40 ng/ml) in the presence of HA (100 μg/ml) at several molecular weights for the indicated times, and whole lysates were subjected to Western blot analysis. *Lane 1*, control; *lane 2*, LMW-HA (M_r = 8,000); *lane 3*, HMW-HA (M_r = 2,000,000); *lane 4*, RANKL (40 ng/ml); *lane 5*, RANKL (40 ng/ml) and LMW-HA (M_r = 8,000); *lane 6*, RANKL (40 ng/ml) and HMW-HA (M_r = 2,000,000).

with RANKL were unchanged in the presence and absence of HA at all time periods tested.

SB203580 Inhibits the Differentiation of RAW 264.7 Cells Induced by RANKL and LMW-HA—To further examine the role of p38 MAPK in RANKL- and HA-mediated osteoclast differentiation, RAW 264.7 cells were treated with RANKL, HA at several molecular weights, or SB203580 (a specific inhibitor of p38 MAPK). A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide viability assay showed that SB203580 had no significant effect on the proliferation of RAW 264.7 cells (data not shown), whereas it inhibited the induction of TRAP-positive multinucleated cells by RANKL and LMW-HA in a dose-dependent manner (Fig. 8). To confirm that p38 MAPK was inhibited by SB203580, RAW 264.7 cells were treated with RANKL and HA at several molecular weights in the presence and absence of SB203580. Western blot analysis revealed that SB203580 inhibited p38 MAPK activity mediated by RANKL and LMW-HA (Fig. 9).

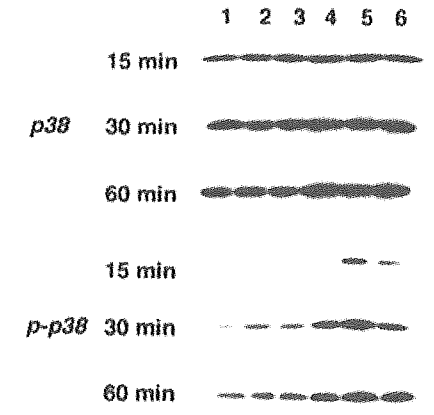

FIG. 7. **Detection of phosphorylated p38 protein in RAW 264.7 cells treated with HA.** RAW 264.7 cells were exposed to RANKL (40 ng/ml) in the presence of HA (100 μg/ml) at several molecular weights for the indicated times, and whole lysates were subjected to Western blot analysis. *Lane 1*, control; *lane 2*, LMW-HA (M_r = 8,000); *lane 3*, HMW-HA (M_r = 2,000,000); *lane 4*, RANKL (40 ng/ml); *lane 5*, RANKL (40 ng/ml) and LMW-HA (M_r = 8,000); *lane 6*, RANKL (40 ng/ml) and HMW-HA (M_r = 2,000,000).

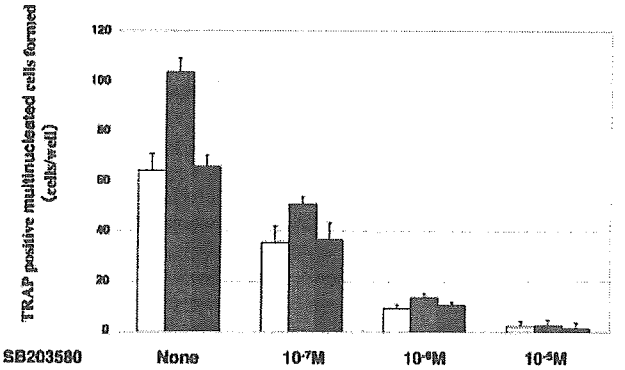

FIG. 8. **Effects of SB203580 on RAW 264.7 cells treated with RANKL and HA.** RAW 264.7 cells were cultured with RANKL (40 ng/ml) and SB203580 in the presence of HA (100 μg/ml) at M_r = 8,000 and 2,000,000. RAW 264.7 cells were stimulated with RANKL (40 ng/ml) in the presence of HA (100 μg/ml) and SB203580 (10^{-5}, 10^{-6}, and 10^{-7} M) for 6 days. After TRAP staining was performed, the number of OCLs was counted. Data are expressed as the mean ± S.D. of triplicate cultures. The experiment was performed three times, with similar results obtained in each experiment. *, $p < 0.05$ (Student's t test). □, RANKL; ▩, RANKL + LMW-HA; ■, RANKL + HMW-HA.

CD44 Function-blocking Monoclonal Antibody Inhibits the Expression of c-Src and RANK Proteins in RAW 264.7 Cells Induced by RANKL and LMW-HA—To further examine the role of CD44 as an HA receptor in this signaling pathway, RAW 264.7 cells were treated with RANKL and LMW-HA in the presence of CD44 function-blocking monoclonal antibody. Western blot analysis revealed that the monoclonal antibody inhibited the enhancement of c-Src and RANK protein expression mediated by LMW-HA (Fig. 10).

DISCUSSION

In this study, we used a homogeneous clonal population of murine monocyte RAW 264.7 cells to elucidate the direct effects of RANKL and HA on osteoclast differentiation and function. This cell line is known to express RANK and to differentiate into TRAP-positive cells when cultured with bone slices and RANKL (21). The main advantage of this system is that it does not contain any osteoblast/bone marrow stromal cells, which may also be targets of RANKL and LMW-HA actions. We

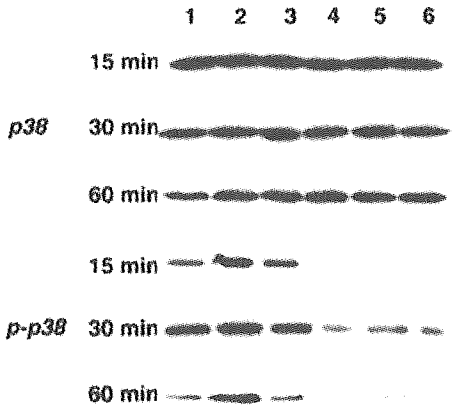

FIG. 9. **Effect of SB203580 on phosphorylated p38 protein in RAW 264.7 cells treated with RANKL and HA.** RAW 264.7 cells were exposed to RANKL (40 ng/ml) and SB203580 (10^{-6} M) in the presence of HA (100 μg/ml) at several molecular weights. Whole cell lysates were subjected to Western blot analysis. *Lane 1*, RANKL; *lane 2*, RANKL + LMW-HA (M_r = 8,000); *lane 3*, RANKL + HMW-HA (M_r = 2,000,000); *lane 4*, RANKL (40 ng/ml) + SB203580; *lane 5*, RANKL (40 ng/ml) + LMW-HA (M_r = 8,000) + SB203580; *lane 6*, RANKL (40 ng/ml) + HMW-HA (M_r = 2,000,000) + SB203580.

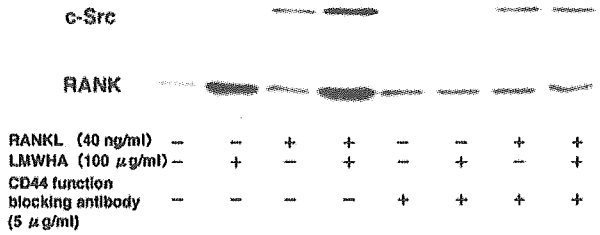

FIG. 10. **Effect of CD44 function-blocking monoclonal antibody on c-Src and RANK proteins in RAW 264.7 cells treated with RANKL and LMW-HA.** RAW 264.7 cells were exposed to RANKL (40 ng/ml) and LMW-HA (100 μg/ml) in the presence or absence of CD44 function-blocking monoclonal antibody BRIC 235 (5 μg/ml). Whole cell lysates were subjected to Western blot analysis.

focused on pre-osteoclast cells to examine the effects of RANKL and LMW-HA on differentiation and function. We also found that LMW-HA activated OCL formation induced by RANKL in mouse bone marrow culture (data not shown). These results suggest that the stimulatory effect of LMW-HA on OCL formation is involved in the RANKL-mediated signaling pathway in mouse bone marrow cells as well as RAW 264.7 cells.

RANKL, a member of the tumor necrosis factor family, triggers osteoclastogenesis by forming a complex with RANK, a member of the tumor necrosis factor receptor family. The binding of RANKL to RANK results in a cascade of intracellular events, including the activation of the intracellular adaptor protein family in pre-osteoclast cells (21–23). Our results show that LMW-HA markedly increases the level of RANK protein expression in RAW 264.7 cells. It is possible that LMW-HA changes RANK action, with an ultimate increase in osteoclast development in addition to increases in RANK production.

Bone resorption is a multistep process initiated by the proliferation of immature osteoclast precursors, which is followed by the commitment of those cells to the osteoclast phenotype and degradation of the organic and inorganic phases of bone by mature resorptive cells. Like their *in vivo* counterparts, *in vitro* generated osteoclasts are capable of bone resorption. When cultured with bone or dentin, osteoclasts excavate resorptive lacunae, or pits, which are similar to the structures formed when the cells degrade bone *in vivo*, and the number and size of the resorption lacunae formed *in vitro* are used as a quantitative measure of osteoclast activity (24). In this study, we used Osteologic™ slides coated with calcium phosphate substrate and observed the up-regulation of the pit-forming activity of OCLs stimulated with RANKL.

RANKL increases the level of c-Src protein, another marker molecule of osteoclast differentiation (25) that is a widely expressed non-receptor tyrosine kinase particularly abundant in platelets and neural tissues (26, 27) and osteoclasts (28, 29). c-Src plays an essential role in osteoclast function, as mice in which the *src* gene has been disrupted show normal osteoclast development, but fail to resorb bone, resulting in osteopetrosis (30). In the present TRAP activity results, the expression of c-Src induced by RANKL was enhanced by LMW-HA, suggesting that the enhanced osteoclast formation by LMW-HA is not caused by stimulating the proliferation of monocyte/macrophage lineage cells, but rather by increasing the number of cells committed to osteoclast lineage, which are responsive to RANKL-mediated terminal differentiation to mature osteoclasts.

MAPK family members are proline-directed serine/threonine kinases that are important for cell growth, differentiation, and apoptosis (31–34) and become activated by phosphorylation of threonine and tyrosine in response to external stimuli. MAPK family members are classified into the ERK, JNK, and p38 MAPK groups, and it is widely accepted that peptide growth factors and phorbol esters preferentially activate ERKs, whereas cellular stress, such as that caused by hyperosmolarity or reactive oxygen species, potently activates JNKs and p38 MAPKs (35–37). A previous study has shown that activation of the p38 MAPK pathway plays an important role in the RANKL-induced osteoclast differentiation of precursor bone marrow cells (38). In this study, RANKL-induced activation of ERKs and p38 MAPK was clearly detected, and LMW-HA enhanced the activities of both kinases in RAW 264.7 cells.

The pyridinyl imidazole SB203580, a specific inhibitor of p38 MAPK (39), has been widely used to investigate the roles of p38 MAPK in the regulation of cell differentiation and function (37, 40, 41). p38 MAPK-mediated signals were shown to be involved in osteoclast bone resorption induced by interleukin-1 and tumor necrosis factor-α in fetal rat long bones using SB203580 (41). These results suggest that p38 MAPK-mediated signals regulate osteoclast differentiation or function. In this study, SB203580, but not PD98059, inhibited differentiation and TRAP activity induced by RANKL and LMW-HA, whereas it inhibited only p38 MAPK activity in RAW 264.7 cells (Fig. 9). Our findings suggest that p38 MAPK may play a critical role in RANKL/LMW-HA-induced osteoclast formation in RAW 264.7 cells. However, we cannot rule out the possible involvement of ERK in osteoclastogenesis.

In our experiments, LMW-HA enhanced both the differentiation and function of RAW 264.7 cells when cultured with RANKL. Interestingly, HMW-HA had almost no effect on osteoclast formation and function. Previous studies (4–11) have confirmed the physiological effects on chondrocytes; however, relatively little attention has been directed toward the differentiation and function of osteogenic cells in bone metabolism. Furthermore, there are no known reports concerning the pathological effects of different molecular weights of HA on osteoclastogenesis or its signal transduction in osteoclasts. Our results suggest that HA at low molecular weights is effective in enhancing osteoclast formation and function under inflammatory conditions (15). We have no ready explanation for this phenomenon, but suggest that it is caused by the interaction of HA and HA-binding protein in joint tissues under pathological conditions.

HA is a major component of synovial fluid and plays impor-

tant roles in the joint cavity. CD44, one of the major HA-binding proteins, is expressed in several human cells, including lymphocytes, alveolar macrophages, and fibroblasts, as well as in several kinds of tumor cells (42). Culty *et al.* (43) demonstrated that mature alveolar macrophages may be involved in regulating HA levels in the lung and suggested that the binding of HA and CD44 leads to the efficient degradation of HA. Furthermore, it has been reported that CD44-mediated degradation of HA may play an essential role during embryonic morphogenesis and the development of some organs, such as the liver, spleen, and thymus; bone marrow; and hair follicles. More recently, Cao *et al.* (44) reported that HA activates CD44 to stimulate RANKL expression in bone marrow stromal cells. In this study, we found that CD44 function-blocking monoclonal antibody remarkably inhibited the effect of HA on the signal introduction of c-Src and RANK in RAW 264.7 cells. On the basis of these findings, we speculate that the expression of CD44 in osteoclasts, which have the same lineage as alveolar macrophages, leads to the localized degradation of HA in the joint cavity, which in turn leads to induction of osteoclast formation and activation mediated by LMW-HA in the surrounding tissues. Further study is needed to examine the correlations between LMW-HA and CD44 regarding the enhancement of osteoclast formation and activation induced by RANKL in bone marrow stromal cells as well as signal transduction during osteoclastogenesis in joint tissue destruction.

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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 ($P < 0·005$) and H_2S-producing bacteria ($P < 0·05$) in the odour group were significantly larger than those in the no/low odour group. Bacteria forming black or grey colonies (126 isolates from the odour group; 242 isolates from the no/low odour group) were subcultured, confirmed as producing H_2S and identified according to 16S rRNA gene sequencing. Species of *Veillonella* (38·1 % in odour group; 46·3 % in no/low odour group), *Actinomyces* (25·4 %; 17·7 %) and *Prevotella* (10·3 %; 7·8 %) were the predominant H_2S-producing bacteria in both the odour and no/low odour groups. These results suggest that an increase in the number of H_2S-producing bacteria in the tongue biofilm is responsible for oral malodour, although the bacterial composition of tongue biofilm was similar between the two groups.

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INTRODUCTION

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

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

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

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

Abbreviation: VSC, volatile sulfur compound.

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

METHODS

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

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

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

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

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

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

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

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

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

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

RESULTS AND DISCUSSION

Relationship between oral malodour level and clinical indicators

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

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

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

Data are presented as mean ± SD.

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

*Determined by gas chromatography.

†Below the detection limit.

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

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

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

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

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

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

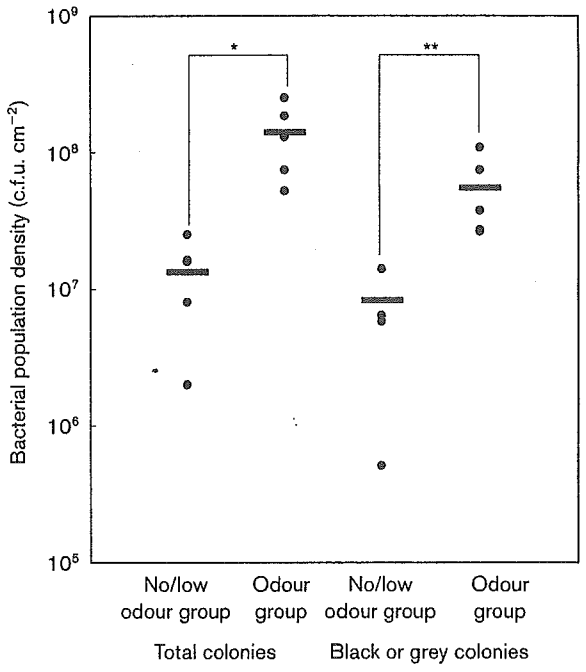

Fig. 1. Numbers of total and black or grey colonies in no/low odour and odour groups. *$P = 0·002$; **$P = 0·012$. Horizontal bars represent means.

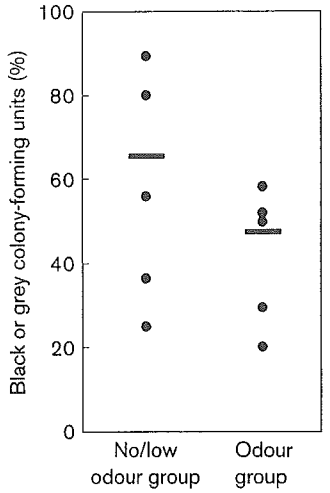

Fig. 2. Proportion of black or grey colony-forming units among total colony-forming units in no/low odour and odour groups. Horizontal bars represent means.

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

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

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

Identification of H_2S-producing bacteria in tongue biofilm

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

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

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

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

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

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

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

†Percentage among total bacterial isolates from each subject.

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

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

Conclusions

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

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編集後記

医療保険および介護保険の改正により、高齢者における口腔ケアや摂食機能療法の積極的な推進が予測されるが、唾液分泌低下や口腔乾燥は、これらのケアやリハビリテーションにとって、障害因子になる。また、口腔乾燥状態は、誤嚥性肺炎の発症や栄養状態悪化とも関連していることが明らかになってきている。

そこで、高齢者の口腔乾燥改善と食機能支援について、平成17年度から総合的な研究を開始し、初年度は、高齢者の口腔乾燥と全身状態や食機能との関連性、改善方法の検討について研究を進め、また、基礎的研究と予防医学的研究についても着手した。その結果、本研究事業で、高齢者の口腔乾燥改善と食機能支援に対する臨床的対応への足がかりになる研究成果が得られた。

本年度は初年度でもあり、口腔乾燥改善と食機能支援における臨床研究と基礎研究を進めたが、来年度は、今年度の研究成果を踏まえて、より臨床的に有用なツールになるような研究成果を出したいと考えている。

本研究事業の研究成果が、高齢者における口腔乾燥や唾液分泌低下の実態を明らかにし、また、口腔乾燥に関連した症状や疾患、摂食機能障害の改善と支援に役立てば望外の喜びである。

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