研究者から見た「未来歯科医学」④

イノベーションの推進のために

松下健二
Kenji Matsushita
国立長寿医療センター研究所
口腔疾患研究部

今回の「Dentistry Quo Vadis? 未来歯科医学に向けて」では，まず1日目に齲蝕と歯周病のサイエンスの現状と未来について，花田信弘氏，高橋信博氏，それに座長の山田 正氏と高添一郎氏などが加わり，熱い討論が繰り広げられた．特に，齲蝕の病因論における mutans streptococci の位置づけに関して意見がぶつかった．齲蝕の病因論に関して学者間で見解が異なり，いまだに十分なコンセンサスが得られていないことが浮き彫りになった．

しかしその一方で，齲蝕原性細菌の一つとして *Streptococcus mutans* を捉え，それを指標として齲蝕対策を講ずることで，齲蝕罹患者数が減ってきたという歴史的な事実がある．そのような意味で，ミュータンス菌病因論の果たした役割は大きいのではないかと思う．また，今回のプレゼンテーションのなかで歯周病に関する話題提供がほとんどなく，歯周病の予防に関する十分な議論がなされなかったことは残念であった．

2日目のインプラント治療の現状と課題に関する講演では，インプラント医学の問題点が浮き彫りにされた．歯科用インプラントの開発と応用は，歯科の発展のために非常に重要であるが，その一方でまだ学問として未成熟の部分があり，そこが医療事故などの問題につながっている．学会主導での治療指針の策定と，それをもとにした学部学生と歯科医師の教育が早急に行われる必要がある．

また，1日目最後の須田立雄氏の講演は，インプラント学の現状を把握するうえで大変有意義なものだった．インプラント体と生体の相互作用などの基礎研究が十分なされておらず，インプラント治療の予後を左右するその機序に関する研究を早急に進める必要がある．

イノベーションの推進のなかに歯科医学の明るい未来がある．インプラント学は未来歯科医学の中核をなすものであり，再生医学の発展と相まってさらに発展していくものと思われる．しかし，その礎が脆弱であっては歯科の未来もおぼつかない．インプラント学の基盤を整備することが喫緊の課題である．そして，予防歯学を基盤とした再建・再生医学の確立こそが明るい歯科医学の未来を切り開く（図1）．

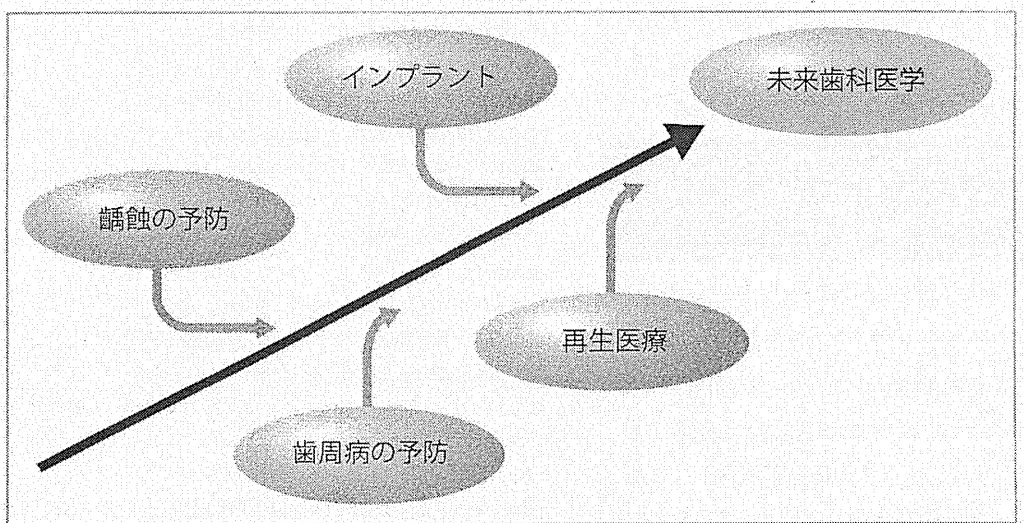

図1 予防歯学を基盤とした効果的な再建・再生歯学の確立

3. ヒストンアセチル化制御薬を用いたHMGB1の放出制御

杉浦　進介，江口　傑徳，小松　寿明，松下　健二

国立長寿医療センター研究所口腔疾患研究部

はじめに：敗血症の死のメディエーター

敗血症は細菌感染を基盤とする全身性炎症反応症候群であり，ショックや多臓器不全に移行する重篤な病態である。敗血症を契機に，本邦では毎年推定5万人以上，米国では毎年約22万人が死亡しており，抗菌剤や抗炎症剤が発達した現在でも，集中治療領域において重大な死因となっている[1]。

1999年，Wangらは High Mobility Group Box 1（HMGB1）が致死的エンドトキシン血症や敗血症のメディエーターであると報告した。彼らは，エンドトキシン血症や敗血症のモデルマウスで血中のHMGB1が発症後16～32時間後に上昇すること，またマウスへの組換えHMGB1投与により発熱や組織破壊が誘導され，敗血症の病態が誘発されることを示した。一方，抗HMGB1抗体やHMGB1阻害剤の投与は，エンドトキシン血症や敗血症マウスの生存率を改善することが明らかにされた[2]。

1. High Mobility Group Box 1 (HMGB1)

HMGB1は，1976年に仔牛の胸腺より同定された215アミノ酸残基から成るタンパク質で，そのC末端側は負電荷の acidic tail，N末端側はそれぞれ約70残基から成る正電荷の“HMG box A，box B”から構成されており，2つの核移行シグナル（Nuclear Localization Signal：NLS）を有している。HMGB1の受容体としては，Receptor for Advanced Glycation End Products（RAGE）やToll Like Receptor（TLR)-2/4が知られている。

HMGB1は，多彩な細胞の核内に普遍的に存在する非ヒストンタンパク質であり，box A，box BによりDNAと親和性を示す。核内ではタンパク質複合体を形成しクロマチン構造の安定性を保持して，さまざまな遺伝子の転写制御に関わっている。HMGB1ノックアウトマウスは，肝臓にグリコーゲンを貯蔵できず，その表現型は野生型に比べ骨格が小さく，脊椎が湾曲し，歩行困難を伴っていた[3]。またその機序としてHMGB1が骨軟骨組織の形成に関わっていることが示唆されている。さらに，HMGB1が組織の修復や，DNA損傷の修復に寄与するとの報告もある[4, 5]。

通常核内に存在するHMGB1であるが，炎症反応時に2通りの機構により細胞外へと放出される。それは，壊死した細胞から放出される passive releaseと，活性化したマクロファージ（Mϕ）や単球，さらには傷害を受けた血管内皮細胞が放出する active secretionである。核内のHMGB1が，細胞質へ移行し，細胞外へ放出されるためにはHMGB1のNLS中のリジン残基のアセチル化が必要である[6]。一度，細胞外に放出されたHMGB1は，RAGEやTLR-2/4などと結合し，炎症性サイトカインとして作用して，炎症反応を促進する。このように，HMGB1は核内のクロマチン構造ではDNAに結合しその修飾因子として機能し遺伝子発現を制御する一方で，ひとたび細胞外へ出ると炎症性サイトカインとして機能したり，組織の修復・再生を制御したりする。

2-1. HMGB1と炎症性疾患

これまで，HMGB1はさまざまな炎症性疾患において検出されてきた。実験動物では組換え

HMGB1の投与により炎症反応が誘発されること，また抗HMGB1抗体によりその病態が軽減されることから各種炎症性疾患（肺水腫，肝炎，肝硬変，アテローム性動脈硬化症，心筋梗塞，脳梗塞，関節リウマチなど）とHMGB1との関連性が示唆されている。

2-2. HMGB1と心血管疾患

アテローム性動脈硬化の病巣部位では高濃度のHMGB1が検出されるが，それは活性化したMφや単球のみならず，傷害された血管内皮細胞からもHMGB1が放出されるためとされている[7]。HMGB1等のサイトカインの作用でMφや血管平滑筋細胞などが病巣部に遊走し，誘導された単球系細胞がさらにHMGB1やサイトカインを放出することで炎症反応が増強され，血栓形成が促進される。

過剰のHMGB1は組織破壊をもたらす一方，適量のHMGB1は組織保護的に働く可能性がある。

3-1. アセチル化修飾

ヒストンのアセチル化などのヒストン修飾が細胞の増殖や分化，遺伝子発現を選択的に調節することが知られている[8]。

例えば，転写共役因子（転写コアクチベーター）の多くは，ヒストンアセチル化酵素（Histone Acetylase：HAT）活性によりヒストンテイルの特定のリジン残基をアセチル化し，ターゲット遺伝子の発現を促進する。一方，ヒストン脱アセチル化酵素（Histone Deacetylase：HDAC）によるヒストンテイルのアセチル基の除去は，ターゲット遺伝子の発現を抑制する。HATやHDACはヒストンのアセチル化の状態を制御するのみならず，非ヒストン核内タンパク質や転写因子のアセチル化の状態も制御することが知られている。例えば，Trichostatin A（TSA）はNF-κBサブユニットのアセチル化を促進し，炎症性遺伝子の発現を亢進する。このように，アセチル化制御薬の効果はその細胞が置かれた状態によりさまざまであり，同時に多様な細胞内シグナル伝達経路に関わる。

3-2. HDAC阻害薬：Valproic Acid

バルプロ酸（VPA）は，1963年に抗てんかん薬としての効果が発見され，現在では，GABA分解酵素阻害剤として，抗てんかん薬，抗躁薬として処方されている。VPAの作用点は，コレプレッサー（Nuclear Receptor Corepressor：NCoR）結合性のHDACsのような普遍的な遺伝子発現調節因子であることが知られる。HDAC阻害剤としてのVPAは，0.5〜2 mMでHDAC1，2，3，4，8を選択的に阻害し，その結果ヒストンH3，H4のアセチル化が起こる[9]。加えて，VPAはクロマチン構造を制御する遺伝子の発現を変化させる。すなわち，VPAは乳癌細胞においてクロマチン構造維持（Structural Maintenance of Chromosomes：SMC）タンパク質，SMC関連タンパク質，DNAメチル化酵素，ヘテロクロマチンタンパク質等を減少させ，その結果クロマチンの脱凝集，DNase感受性の亢進，層状のDNA間に挿入されるインターカレーター（アドリアマイシンなどの抗がん剤）とDNAとの相互作用が亢進される。

4. アセチル化制御薬によるHMGB1の放出制御

HMGB1の細胞外放出には，NLS中のリジン残基のアセチル化を伴うことが報告された[6]。そこでわれわれは，ヒストンのアセチル化を制御するHDAC阻害薬とHAT阻害薬に着目し，HDAC阻害薬であるVPAとHAT阻害薬Anacardic acid（AA）を用いて，Mφ系細胞株RAW-Blue CellにおけるHMGB1の動態制御を試みた。

RAW-Blue Cellの培養上清中HMGB1濃度はVPA添加で無添加群に比べ4.2倍に増加した。VPAによるHMGB1の放出にはHMGB1 mRNAの発現変動を伴わないこと，また細胞内HMGB1濃度の減少することが明らかになった。以上の結果より，VPAは，核内に既存のHMGB1を細胞外へと放出することが証明された。

さらに，HAT阻害剤AAをVPAと同時添加し，HMGB1の動態を検討した。その結果VPAとAAの同時添加により，VPA単独添加に比べ23%，HMGB1の放出が抑制された。すなわち，

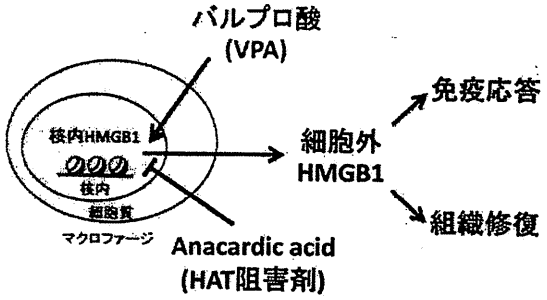

図 1　アセチル化制御薬による HMGB1 の放出制御
HDAC 阻害剤であるバルプロ酸（VPA）により，マクロファージ様細胞から HMGB1 の放出が誘導された。一方，HAT 阻害剤である Anacardic acid（AA）により HMGB1 の放出が抑制された。細胞外に放出された HMGB1 は，免疫応答を割賦する一方，組織修復を促進する可能性がある。

HDAC 阻害剤 VPA で促進された HMGB1 の放出は，HAT 阻害剤 AA で抑制されるという結果が得られた。

まとめ

今回われわれは，敗血症の死のメディエーターである HMGB1 の放出制御を目的として研究を行った。その主たる結果として HDAC 阻害剤 VPA により，Mφ 系細胞から HMGB1 が放出された一方，HAT 阻害剤 AA により HMGB1 の放出が抑制された。この事実はアセチル化制御薬によって HMGB1 の放出制御ができる可能性を示している（図 1）。

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鹿児島大学医学雑誌　第61巻　第3号　47-51頁　2010年1月
Med. J. Kagoshima Univ., Vol. 61, No. 3, 47–51, January, 2010

高齢者の残存歯数と認知機能との関連性

今井　剛[1)]，西永　正典[3)]，松下　健二[2)]

[1)] 国立長寿医療センター研究所老化制御研究部
[2)] 同口腔疾患研究部
[3)] 高知大学医学部老年病科

（原稿受付日　2009年10月26日）

Association of Number of Teeth with Cognitive Function in the Elderly

Takeshi IMAI[1)], Masanori NISHINAGA[3)], Kenji MATSUSHITA[2)]

Department of Aging Intervention[1)], Department of Oral Disease Research[2)], National Institute for Longevity Science, National Center for Geriatrics and Gerontology, Aichi, Japan
[3)] Department of Medicine and Geriatrics, Kochi Medical School, Kochi, Japan

Abstract

It has been reported that number of teeth is associated with cognitive function in elderly populations with dementia. However, little is known about this association in an ordinary elderly population. We evaluated this relationship in a Japanese population of elderly people aged from 65 to 92 years (n = 345; 122 males and 223 females) residing in Kahoku-chou (now Kami City) in Kochi Prefecture of Japan. Dental examinations were performed all subjects with the Mini-Metal State Examination (MMSE) and Kohs task test for assessing cognitive function. Associations were not found between number of residual teeth and MMSE in total subjects or in males or females. However, associations were found between number of residual teeth and Kohs score in males. These results suggest that cognitive functions, especially, motor cognition, may be associated with number of teeth in ordinary elderly males.

Key words: cognitive function, dementia, teeth, oral health, elderly, longevity

緒　言

高齢者における認知機能の低下やうつ状態は，高齢者の生活の質（Quality of Life; QOL）を著しく低下することが指摘されており，今後我が国において高齢化社会が進行するにあたって，認知機能の改善，認知機能低下の予防対策は急務である．

最近の疫学調査の結果から，自分の歯でよくかむことは高齢者の栄養状態の維持に重要であるばかりでなく，全身状態にも良い影響を及ぼしていることが解明されつつある．我が国においては，1989年に厚生省（現厚生労働省）が“80歳で自分の歯を20本保持しよう”という8020運動が提唱され，自分の歯を残す意義について検証が始まった．厚生労働省も1997年度から厚生科学研究事業の1テーマとして“高齢者の口腔保健と全身的な健康状態の関係についての総合研究”を課題に掲げ，福岡県，

Corresponding author: Kenji Matsushita, DDS, PhD
Department of Oral Disease Research[1)],National Institute for Longevity Science, National Center for Geriatrics and Gerontology36-3 Gengo, Morioka-cho, Obu, Aichi 474-8511, Japan
E-mail: kmatsu30@nils.go.jp

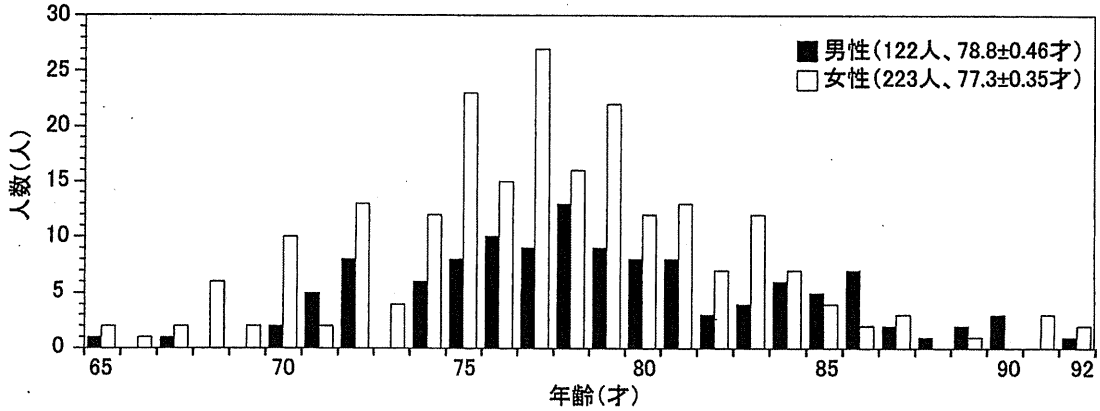

図1．被験者の年齢分布．65歳から92歳までの男性（122人，平均年齢78.8±0.46才），女性（223人，平均年齢77.3±0.35才）について，横軸に年齢を，縦軸にその人数を示した．

愛知県，新潟県などで疫学研究が実施された．それらの研究の結果から，歯・咀嚼機能とQOL，咀嚼機能と日常生活動作（Activities of Daily Living; ADL），咀嚼機能と運動能力，咀嚼機能と認知能力等との関連性が明らかになった[1]．歯の喪失がAlzheimer病（AD）発症の危険因子の一つであるとの報告があり，ADでは残存指数が少ないことが指摘されている[2,3]．また，咬合力と認知能力との正の相関も指摘されてきた[4]．簡易認知機能検査法である Mini-Mental State Examination (MMSE)[5]を用いた，福岡県において1998年に始まった疫学調査の結果では，60歳および65歳住民の残存歯数とMMSEとの間に有意な正の相関が認められている[6]．以上の結果は，残存歯数と認知能に関連性があることを示唆している．

本研究では，高知県香北町において1991年から2001年にかけて行われた高齢者の長期縦断疫学調査の調査結果をもとに[7-23]，高齢者の残存歯数と認知機能，特に動作性認知機能との関連性について検討した．

対象および方法

本研究は，1991年から2001年にかけて高知県香北町において行われた縦断的検診事業「香北町健康長寿研究」(KAHOKU LONGITUDINAL AGING STUDY; "KALS")の調査結果の内1994年度に行われたデータを統計学的に解析したものである[7-23]．

対象者は，同町在住の65歳以上の老年者1,488名のうち，残存歯数検査および簡易板Kohsテスト，MMSEテスト等の認知機能テストを受けた被験者で，低酸素性脳症，脳卒中後遺症等については除外した345名（男：女＝122：223)である(図1)．平均年齢＋標準誤差は男性78.8±0.46才で，女性は77.3±0.35才である．特に，簡易版Kohs立方体テストに関しては，今回は時間を短縮し効率を上げるために，原版の課題1，2，4，7，10，11，14の7題を選択して実施した（47点満点)[7]．なお，KALSにおける簡易版Kohsテストでも認知機能を評価できることが既に確認されている[7,10,12]．

統計学的解析にあたっては，分散分析（analysis of variance; ANOVA)を用いて解析を行った．有意差のあるものに関してはFisher's exact testを用い，またp値をSTATVIEWを用いて計算した．有意水準はp<0.05とした．図2から図4の棒グラフには平均値と標準誤差を示した．

結　果

1．残存歯数と年齢，性差との関係

図2に，年齢と残存歯数の関係を示した．65歳から92歳の被験者を4つの群（A群；65-74才，B群；75-80才，C群；80-85才，D群；86-92才）にわけ，男女別に残存歯数の平均値を算出した．その結果，男性においてはA群（65-74才)とD群（86-92才）に有意な差がみられた(p=0.0080)．女性においてはA群（65-74才）に対してC群（81-85才）及びD群（86-92才）との間に有意差がみられた（共にp<0.0001)．

更には，1993年度の厚生労働省口腔疾患実態調査のデータ（70～74才：14.41本，75～79才：9.01本，80～84才：7.41本)[24]と高知県香北町における高齢者の歯の残存歯数のデータと本調査による高知県香北町のデータ(70～74才：8.49本，75～79才：6.69本，80～84才：3.64本）を比較すると，前期高齢者，後期高齢者とも全国平均を下回っていた．

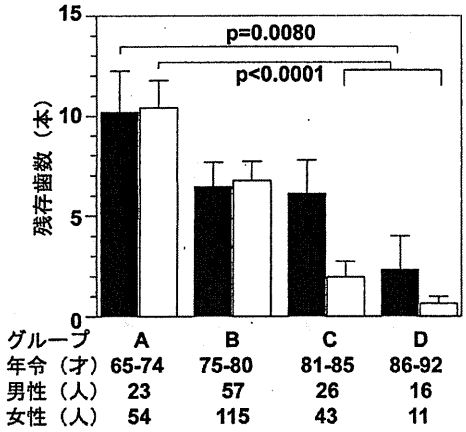

図2．性別高齢者年齢と残存歯数の関係．年齢別に4群（A, B, C, D）に分けて，各群における残存歯数の平均値および標準誤差を示した．A群：65-74才（男性23人, 女性54人），B群：75-80才（男性57人, 女性115人），C群：81-85才（男性26人,女性43人），D群：86-92才（男性16人,女性11人）．A群男性に比べてD群男性は有意に低く（p=0.0080），A群女性（レーン2）に比べてC, D群の女性（レーン8, 10）は共に有意に低かった（p<0.0001）．

2．残存歯数と認知機能の関係

次に，認知機能と残存歯数との関連性について，MMSEと簡易版Kohs立方体テスト等と残存歯数との関連を解析した（図3）．図2の結果より加齢により（男性D群，女性CおよびD群）平均残存歯数が有意に下がるため，男性・女性ともに有意差がみられない65歳から80歳に設定した（男性80人，平均年齢75.8±0.37才，女性169人，平均年齢75.2±0.27才）．歯がない男性は32人（平均年齢76.9±0.45才），歯がある男性は48人（平均年齢75.1±0.48才），歯がない女性は80人（平均年齢76.2±0.35才），歯がある女性は89人（平均年齢74.4±0.36才）であった．歯のある男性の平均歯数は12.5±1.44本で，歯のある女性の平均歯数は15.2±1.07本であった．

歯の有無と簡易版Kohsスコアとの関連性を調べた結果，男性にのみ有意差が認められ，歯のある65-80歳の男性は歯のない男性に比べKohsスコアが有意に高かった（p=0.0252）．一方，女性に関しては歯の有無と簡易版Kohsスコアとの間には有意な差は認められなかった（p=0.1008）（図3A）．また，男性群において年齢，MMSEスコアと歯の有無との関連を調べた結果，歯のある男性と歯のない男性の平均年齢には有意差がみられないこと（図3B，各々p=0.1190）さらにはMMSEスコアと歯の有無との間にも有意な相関関係は認められなかった（0.1039）．従って，高齢者の男性において歯の有無と簡易板Kohsスコアとの間に特異的に有意差が見られることが明らかになった．

最後に各個人の数値の相関を解析した（図4）．MMSEと残存歯数（図4B）との間ならびに年齢と残存歯数（図4C）との間には有意な相関はみられなかった．同様の解析をKohsスコアについても行った（図4A）．その結果，図3と同様，歯のない群に比べて歯のある群はKohsスコアが高い傾向にあったが，残存歯数との間に有意な相関関係は認められなかった．

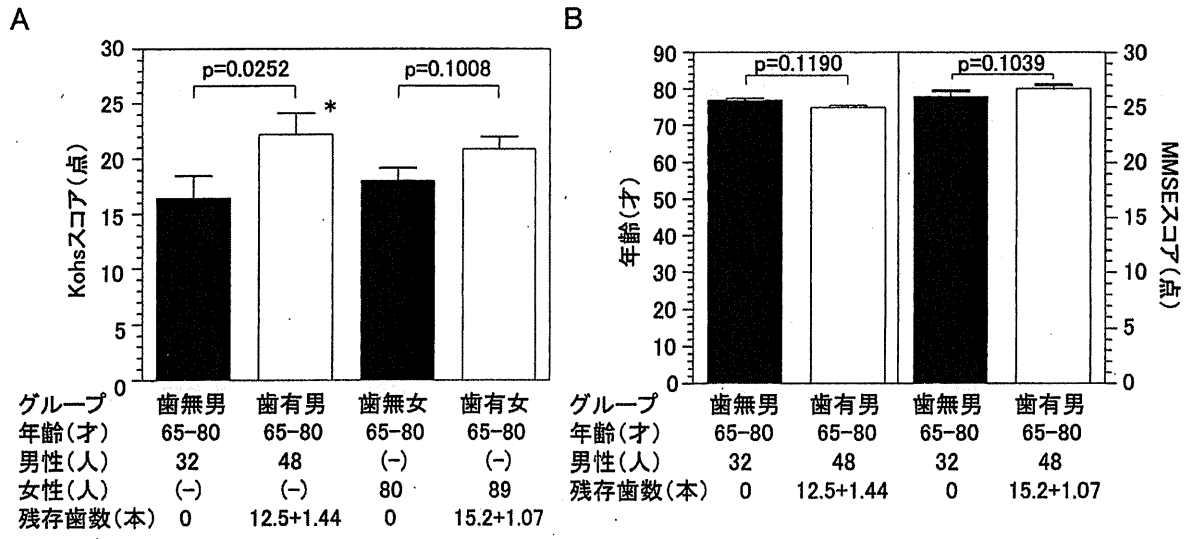

図3．認知機能と歯の有無の関係．65-80歳の男女について簡易版Kohsテスト（パネルA），年齢およびMMSEテスト（パネルB）を行った．歯のない群とある群に分けて，その平均値および標準偏差を表した（歯のない男性32人，歯のある男性48人で残存歯数12.5±1.44本，歯のない女性80人，歯のある女性89人で残存歯数15.2±1.07本）．A）Kohsスコアに関しては男性のみ歯の有無で有意差がみられた（*, p=0.0252）．女性はみられなかった（p=0.1008）．B）年齢およびMMSEスコアに関しては有意差がみられなかった（各々p=0.1190, 0.1039）．

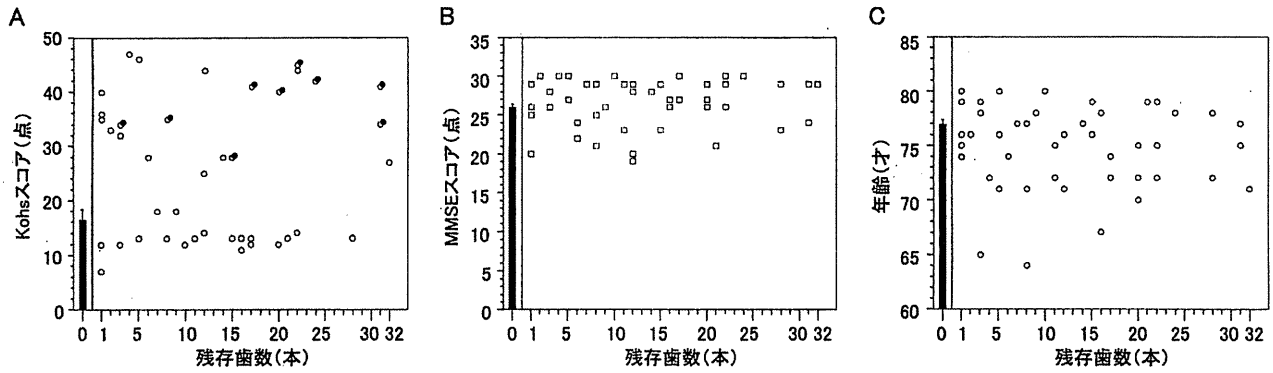

図4．認知機能と残存歯数の関係．65-80歳の男女についてKohsテスト（パネルA），MMSEテスト（パネルB），年齢（パネルC）を行った．残存歯数とKohsスコアをプロットし，両者の相関関係を示した．特に歯のない群の値は棒グラフにすることで歯のある群と差別化した．

考　察

高齢者の歯の残存歯数に関しては，1993年度の厚生労働省口腔疾患実態調査のデータと本調査による高知県香北町のデータを比較すると，前期高齢者，後期高齢者ともに全国平均を大きく下回っており，高齢者口腔の健康状態は良好とは言いがたいことが判明した．同地域におけるオーラルヘルスプロモーションの推進が必要である可能性が考えられた．

認知機能を調べるために，香北町の調査ではMMSE，長谷川式簡易知能評価スケール改訂版（Revised Hasegawa Dementia Scale; HDSR），簡易版Kohs立方体テスト等を行っている．本研究では，MMSEスコアあるいはKohsスコアと残存歯数の関連性について検討した．その結果，残存歯の有無とMMSEスコアとの間には相関はみられなかったものの，残存歯の有無とKohsスコアとの間には有意な相関が認められた．MMSEは，記憶，見当識，計算能力などを質問形式で行うもので，総合的な認知機能を評価することができる．これまでも，健全歯数あるいは残存歯数とMMSEスコアとの有意な相関が示されている[6,26,27]．本研究においても歯の健康状態を考慮して関連性を検討すれば，関連性が浮き彫りになった可能性が考えられるが，本調査では齲蝕の程度について解析がなされていなかったため，相関関係を明らかにすることができなかった．一方，Kohs立方体テストの結果と健全歯数あるいは残存歯数等との関連性を調べた報告はこれまでにない．Kohs立方体テストは，4種の色に塗り分けられた約3cm立方の積み木を4個から16個使い，指示された図版の模様と同じ模様になるように積み木を組み合わせていくものであるため，空間および運動認知機能に特化した試験であるといえる．本研究の結果から，高齢の男性において歯の有無は空間や運動認知機能に影響を及ぼす可能性が示唆された．一般に，認知機能は男性の方が女性より高いことが知られており，これは男性の方が高度な教育を受けていることによることが推察されているが，今回男性に限定して歯の有無と認知機能に相関がみられたことは非常に興味深い．また，咬合関係や咀嚼能力等との関係も非常に興味のあるところであり，今後別の疫学調査において検討していきたいと考えている．

結　論

高齢者の残存歯の有無は高齢者の空間認知機能および運動性認知機能と相関があることが明らかになった．

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Biochemical and Biophysical Research Communications 387 (2009) 42–46

Contents lists available at ScienceDirect

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Attenuation of LPS-induced iNOS expression by 1,5-anhydro-D-fructose

Xiaojie Meng [a,1], Ko-ichi Kawahara [a,1], Kenji Matsushita [b], Yuko Nawa [a], Binita Shrestha [a], Kiyoshi Kikuchi [a], Hisayo Sameshima [a], Teruto Hashiguchi [a], Ikuro Maruyama [a,*]

[a] Department of Laboratory and Vascular Medicine Cardiovascular and Respiratory Disorders Advanced Therapeutics, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan
[b] Laboratory of Oral Disease Research, National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, Aichi 474-8522, Japan

ARTICLE INFO

Article history:
Received 16 June 2009
Available online 24 June 2009

Keywords:
1,5-anhydro-D-fructose
iNOS
IL-10
Acute lung inflammation

ABSTRACT

1,5-anhydro-D-fructose (1,5-AF), a monosaccharide formed from starch and glycogen, exhibits antioxidant and antibacterial activity, and inhibits cytokine release by attenuating NF-κB activation in LPS-stimulated mice. The present study examined whether 1,5-AF inhibits lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) *in vitro* and *in vivo*. We found that 1,5-AF significantly blocked the production of NO, and protein and mRNA expression of iNOS, and up-regulated IL-10 production *in vitro*. We also investigated the effects of 1,5-AF on acute lung inflammation in C57BL/6J mice. We found that protein and mRNA expression of iNOS in lung tissues were inhibited by 1,5-AF pretreatment. In addition, the serum level of IL-10 was upregulated by 1,5-AF. Collectively, the iNOS transcriptional and translational inhibitory effects of 1,5-AF seem to be prolonged and enhanced by the production of IL-10. These results suggest that 1,5-AF could be a useful adjunct in the treatment of acute lung inflammation.

Introduction

Inflammation is a central feature of many pathophysiological conditions in response to tissue injury and host defense against invading pathogens [1]. Macrophages are the main proinflammatory cells involved in the responses to invading pathogens and release many proinflammatory molecules, including nitric oxide (NO). Excessive NO production has been implicated in the pathogenesis of inflammatory tissue injury and in several disease states [2,3]. In activated macrophages, the transcriptionally expressed inducible nitric oxide synthase (iNOS) is responsible for the prolonged enhanced production of NO. Thus, pharmacological inhibition of NO production offers promising targets for therapeutic intervention in inflammatory disorders.

1,5-anhydro-D-fructose (1,5-AF) is a newly identified monosaccharide that is formed directly from starch or glycogen through an α-1,4-glucan lyase reaction (EC 4.2.2.13). During its formation, the carbonyl group does not undergo hemiacetal bonding, but it is instead fully hydrated in aqueous solution so that it may play a metabolically active role [4]. 1,5-AF has been found in fungi [5], red algae [6], *Escherichia coli* [7] and rat liver tissue [8]. 1,5-AF is likely to act as an antioxidant for scavenging reactive oxygen species (ROS) induced by phorbol myristate acetate (PMA) in THP-1 cells, copper-mediated LDL oxidation [9,10], or as antimicrobial agents [10], and can attenuate NF-κB activation [11]. ROS and NO, a reactive nitrogen species (RNS), are believed to be important mediators that lead to lung injury [12] .

Interleukin (IL)-10 is a cytokine that has important anti-inflammatory and antiproliferative properties, and attenuates the severity of various disease states. Furthermore, IL-10 suppresses cellular production of NO, a molecular signal in the inflammatory process, and down-regulates the expression of iNOS, which is regulated as a transcription factor of NF-κB activation in macrophages during acute lung injury [13]. Thus, increased IL-10 levels are required for attenuation of inflammation.

In this study, we investigated whether 1,5-AF affects NO production via its anti-inflammatory activity. We conducted this study to explore the anti-inflammatory effects of 1,5-AF on iNOS expression in lung tissues from C57BL/6J mice and in the murine macrophage cell line RAW264.7, which can be stimulated with LPS to mimic a state of infection and inflammation [14].

Material and methods

Cell culture and treatment. The murine macrophage-like RAW264.7 cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Hyclone Logan, UT). The cells were pretreated with

* Corresponding author. Address: Department of Laboratory and Vascular Medicine, Field of Cardiovascular and Respiratory Disorders, Department of Advanced Therapeutics, Kagoshima University Graduate School of Medical and Dental Science, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan. Fax: +81 99 275 2629.
E-mail address: rinken@m3.kufm.kagoshima-u.ac.jp (I. Maruyama).
[1] These authors contributed equally to this work.

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doi:10.1016/j.bbrc.2009.06.108

1,5-AF (0–500 μg/ml) for 2 h and stimulated with LPS (500 ng/ml; O111:B4, Alexis Biochemical, San Diego, CA) in serum-free Opti-MEM-I medium (Invitrogen, Carlsbad, CA) for various durations. Cells were extracted for iNOS experiments, and the supernatant was collected for NOx measurement.

Measurement of nitrite. The measurement of NOx (NO^{2-} + NO^{3-}) in the supernatant was performed according to the method of Misko et al. [15] with minor modifications. In brief, 2,3-diaminonaphthalene (DAN) was dissolved in 0.62 N HCl at a concentration of 0.05 mg/ml. NO^{3-} in culture medium was reduced to NO^{2-} with nitrate reductase (14 mU) and NADPH (40 μM) at room temperature (RT) for 5 min. The media were then collected and aliquots of each sample (100 μl) were placed into 96-well plates. DAN (10 μl) was then added to each well at RT. After 10 min, 5 μl of 2.8 N NaOH was added to each well, and the plate was read on an Appliskan luminescence spectrometer (excitation 360 nm, emission 440 nm) (Thermo Fisher Scientific, Waltham, MA). Standard curves were made with concentrations of sodium nitrite ranging from 0.04 to 10 μM in phenol red-free DMEM.

Western blot analysis. As described previously [16], RAW264.7 cells were washed in ice-cold PBS, lysed with lysis buffer (0.5 M Tris–HCl, 10% SDS, 10% 2-mercaptoethanol, and 20% glycerol). Next, 30 μg/ml of protein was subjected to SDS–PAGE and then transferred to nitrocellulose membranes (Whatman, Cassel, Germany). The membranes were blocked with 5% non-fat dried milk in Tris-buffered saline containing 0.04% Tween 20 (TBST) and incubated with iNOS antibodies (Ab) (Upstate Inc., Lake Placid, NY) or anti-β-actin Ab (Santa Cruz Biotechnology, Santa Cruz, CA) in TBST supplemented with 1% non-fat dried milk. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary Abs (MP Biomedicals, LLC, Santa Ana, CA) diluted to 1:3000 in TBST supplemented with 2.5% non-fat dried milk. Immunoreactive proteins were detected with an enhanced chemiluminescence detection system (Amersham Biosciences).

Flow cytometric assessment of cell viability. RAW264.7 cells were collected and fixed with 70% ethanol at −20°C for 20 min. After washed with phosphate-buffered saline (PBS), the cells were centrifuged and stained with propidium iodine (PI) solution (PI 20 μg/ml and RNase 625 μg/ml in PBS) for 20 min in the dark. The PI fluorescence was measured with an Epics XL flow cytometer (Beckman Coulter, High Wycombe, Bucks, UK).

Animal studies and treatment protocol. As described previously [17], 7-week-old, male C57BL/6J mice were obtained from Kyudou (Kumamoto, Japan). Animal protocols were approved by the Frontier Science Research Center, Kagoshima University and were conducted according to National Institutes of Health (NIH) guidelines. The mice were housed in a pathogen-free environment under controlled light and humidity conditions, and were provided food and water *ad libitum*. Mice were divided into four groups and treated with: (1) saline solution, (2) 1,5-AF, (3) LPS, and (4) 1,5-AF and LPS ($n = 6$ per group). Mice were given an intraperitoneal (i.p.) injection of LPS (2 mg/kg, Sigma, O55:B5, 1×10^6 EU/mg) or saline, immediately after i.p. injection of 1,5-AF (38.5 mg/kg body weight) or saline. Four hours after the injection, blood was drawn by intracardiac penetration and collected in capillary blood collection tubes (Terumo, Tokyo, Japan). Serum was collected and stored at −80 °C. Lung tissue was obtained immediately after the mice were killed and fixed in 10% neutral-buffered formalin (Nacalai Tesque, Inc, Kyoto, Japan).

RT-PCR. As described previously [17], total RNA was extracted from RAW264.7 cells or lung tissues of mice using an RNAqueous kit (Ambion, Inc., Texas). RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA). The relative mRNA expression levels were determined using an Applied Biosystems 7300 Real-Time PCR System with a TaqMan Universal PCR Master Mix (Applied Biosystems) and NOS2 primers (Mn00440485_ml). The expression levels were calculated as the ratio of the mRNA level for a given gene relative to the mRNA level for glyceraldehyde-3-phosphate dehydrogenase (Mm99999915_ml) in the same cDNA sample.

Immunohistochemistry. Paraffin-embedded 5-μm-thick lung sections were deparaffinized and dehydrated. Antigen retrieval was performed using antigen-unmasking solution (Vector Laboratories Inc., Burlingame, CA). Slides were blocked using Block ACE™ (Dainippon Sumitomo Pharma Co., Osaka, Japan) and incubated with rat monoclonal anti-macrophage Ab (1:100 dilution; Abcam, Tokyo, Japan) or rabbit anti-iNOS polyclonal Ab (1:100 dilution; Santa Cruz Biotechnology) at 4 °C in PBS containing 1% bovine serum albumin. Slides were washed with TBST and incubated with Histofine Simple Stain Mouse MAX-PO (Nichirei, Tokyo, Japan). The slides were washed and stained with 3,3′-diaminobenzidine (DAB; Dako Envision Kit, Glostrup, Denmark). Counterstaining was performed with hematoxylin.

Statistical analysis. Data are expressed as means ± SE. Differences between means were evaluated using unpaired two-sided Student's *t*-test ($P < 0.05$ was considered significant).

Results

1,5-AF inhibits LPS-induced NO production in RAW264.7 cells

Murine macrophage-like RAW264.7 cells are commonly used to investigate anti-inflammatory responses [14]. To investigate

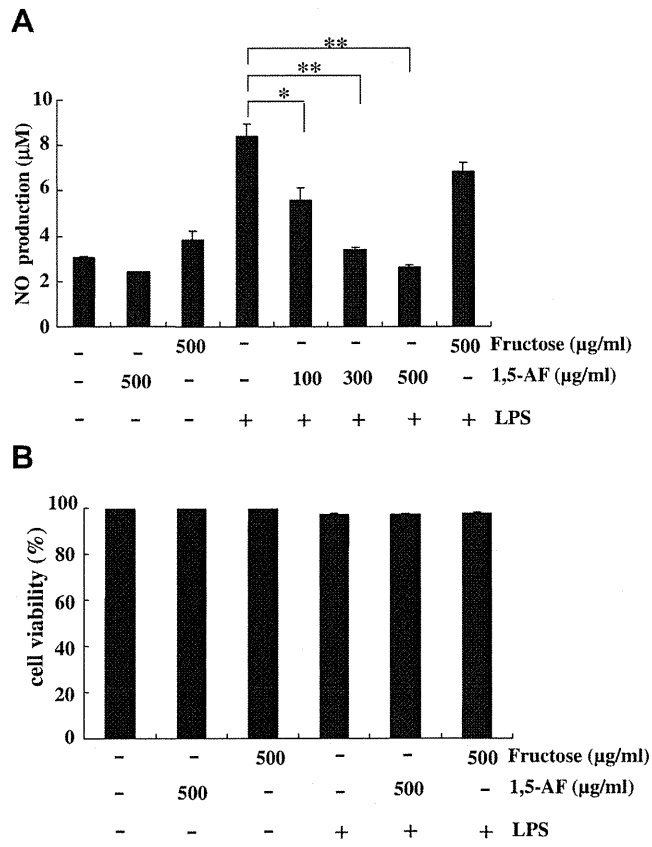

Fig. 1. 1,5-AF inhibits LPS-induced NO production in RAW264.7 cells. Cells were pretreated for 2 h with the indicated concentration of 1,5-AF and were then stimulated with LPS (500 ng/ml) for 18 h. (A) The culture media were collected and assayed for nitrite production. (B) Cells were collected and assayed for cell viability by flow cytometry. The values are expressed as means ± SE of triplicate experiments. *$P < 0.05$ and **$P < 0.01$ indicate statistically significant differences versus the control group.

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whether 1,5-AF suppresses NO production in LPS-treated RAW264.7 cells, RAW264.7 cells were preincubated with 1,5-AF (0–500 μg/ml) or fructose (500 μg/ml) as an analog of 1,5-AF, and were then stimulated with LPS for 18 h. As shown in Fig. 1A ,1,5-AF significantly suppressed NO production compared with that in cells stimulated with LPS alone ($P < 0.01$, $P < 0.05$, Fig. 1A) in a dose-dependent manner; however, fructose had no effect on LPS-induced NO production (Fig. 1A).

To exclude the potential for cytotoxic activity of 1,5-AF, we examined whether 1,5-AF affects cell viability with flow cytometry. As shown in Fig. 1B, the cell viability was not affected by 500 μg/ml 1,5-AF, a concentration that significantly inhibited LPS-stimulated production of NO (Fig. 1A).

1,5-AF inhibits LPS-induced iNOS protein and gene expression in RAW264.7 cells

To investigate whether the inhibition of NO production is caused by reduced iNOS protein expression, Western blot analysis was performed on the LPS-treated lysates with or without 1,5-AF pretreatment. As shown in Fig. 2 A, 1,5-AF pretreatment also inhibited iNOS protein expression in a dose-dependent manner. Expression of β-actin was also determined in the same blot as the loading control and was noted as a consistent band. Furthermore, we performed RT-PCR analysis for iNOS mRNA. As shown in Fig. 2B, 1,5-AF treatment significantly inhibited the expression of iNOS mRNA in LPS-stimulated RAW264.7 cells compared with LPS alone in a dose-dependent manner (LPS alone: 1.06 ± 0.029; 300 μg/ml 1,5-AF: 0.810 ± 0.08, $P < 0.05$; 500 μg/ml 1,5-AF: 0.681 ± 0.08, $P < 0.01$). However, the use of fructose as an analog had no effect on the expression of iNOS mRNA (0.880 ± 0.067).

1,5-AF suppresses LPS-induced pulmonary iNOS expression and mRNA production in C57BL/6J mice

iNOS is a important molecule in lung tissue injury and is primarily expressed by activated macrophages [18]. Thus, to confirm the above results, we examined whether 1,5-AF suppressed iNOS expression in the LPS-challenged C57BL/6J mice. As shown in Fig. 3B and C and analyzed in Supplementary Fig. 1A, 1,5-AF pretreatment for 2 h significantly suppressed iNOS expression in the pulmonary parenchyma in LPS-challenged C57BL/6J mice compared with LPS alone (58 ± 1% vs. 23 ± 0.2%, $P < 0.01$). Few iNOS-positive parenchymal cells were found in the control mice or in the mice treated with 1,5-AF alone (Fig. 3A and D). iNOS expression was greater in specimens obtained from mice exposed to LPS alone than in those obtained from 1,5-AF- and LPS-treated mice. Similar results were obtained from real-time PCR analysis of iNOS mRNA in lung tissue. iNOS mRNA was up-regulated in mice exposed to LPS, whereas pretreatment with 1,5-AF decreased LPS-induced iNOS mRNA expression (Supplementary Fig. 1B; $P < 0.05$ compared with LPS-challenged mice).

1,5-AF increases IL-10 levels in RAW264.7 cell and in C57BL/6J mice

IL-10 also exerts an anti-inflammatory role in acute lung inflammation [13]. iNOS expression in macrophages is down-regulated by IL-10 during acute lung injury [13]. Therefore, we next investigated whether 1,5-AF alters the production of IL-10. RAW264.7 cells were preincubated with 1,5-AF (0–500 μg/ml) for 1 h and then challenged with LPS. There was a significant dose-dependent increase in IL-10 production (Fig. 4A). Furthermore, in LPS-challenged C57BL/6J mice (Fig. 4B), the IL-10 levels were in-

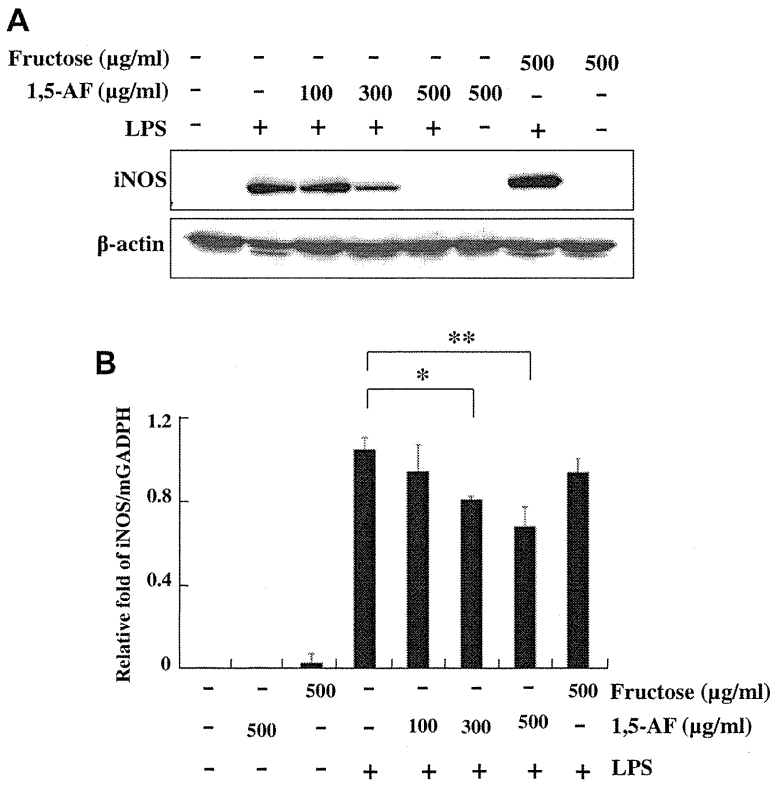

Fig. 2. 1,5-AF inhibits LPS-induced iNOS protein levels and mRNA expression in RAW264.7 cells. Cells were pretreated for 2 h with the indicated concentration of 1,5-AF and were then stimulated with LPS (500 ng/ml). (A) Whole-cell lysates were prepared after 12 h of stimulation, and were analyzed for the presence of iNOS using Western blotting with anti-iNOS Ab. The blot was stripped of the bound Abs and reprobed with β-actin to confirm equal loading. (B) Total RNA was extracted after stimulation for 6 h. iNOS and GAPDH mRNAs were measured by real-time RT-PCR. iNOS mRNA levels were normalized against GAPDH. The values are expressed as means ± SE of triplicate experiments, with four samples per group. *$P < 0.05$ and **$P < 0.01$ indicate statistically significant differences versus the control group.

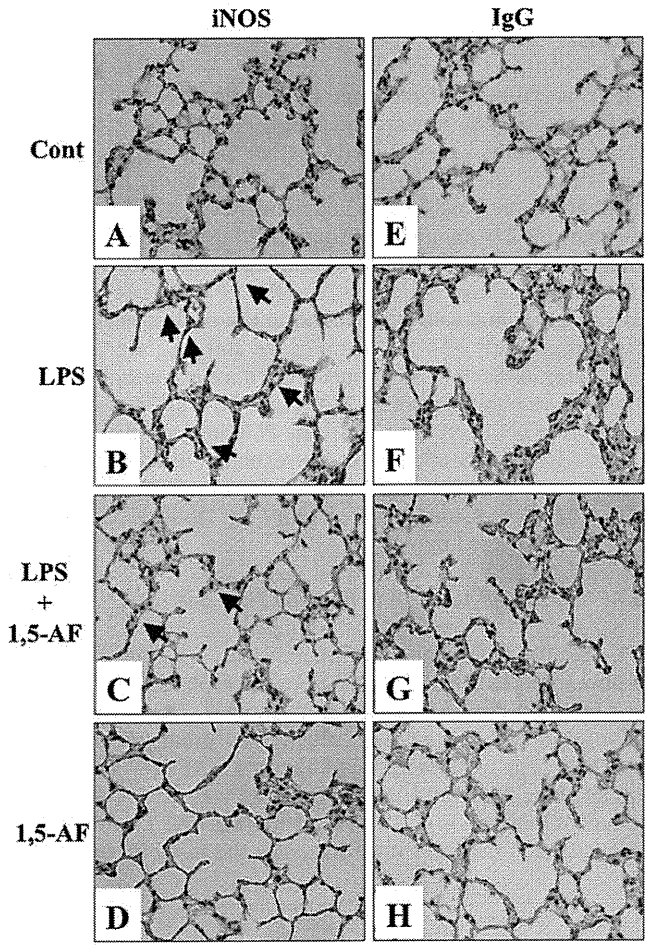

Fig. 3. 1,5-AF inhibits LPS-induced iNOS expression in the lung tissue of C57BL/6J mice. Seven-week-old male mice were challenged for 4 h with either saline alone (A) or LPS (B) immediately after 1,5-AF (38.5 mg/kg) (C) or saline treatment and 1,5-AF alone (D). The lung tissues were stained with iNOS Ab (A–D) and isotype IgG (E–H). The brown regions indicate the DAB-positive area (arrows). Original magnification: 400×.

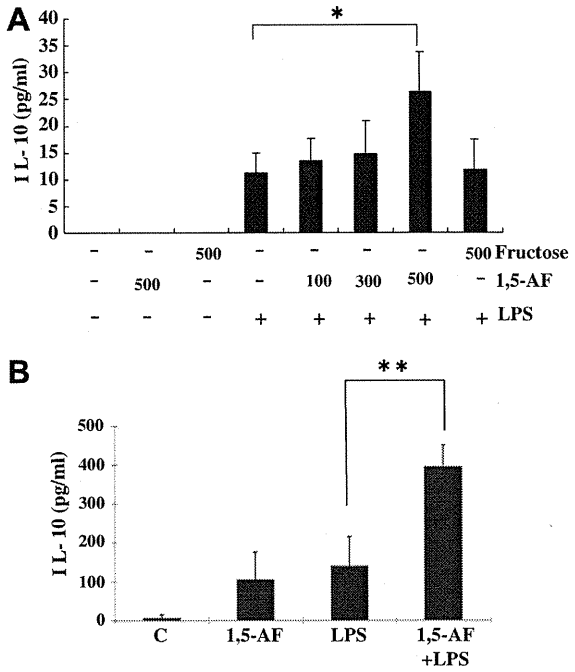

Fig. 4. 1,5-AF upregulates IL-10 production in LPS-stimulated RAW264.7 cells and mice serum. (A) Cells were pretreated for 2 h with the indicated concentration of 1,5-AF and were then stimulated with LPS (500 ng/ml) for 6 h. The concentration of IL-10 released into the supernatant was measured by ELISA. The values are expressed as means ± SE of triplicate experiments. (B) Mice were treated as described in Fig. 3. The serum IL-10 concentrations were measured by ELISA. The values are expressed as means ± SE with six mice per group. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate statistically significant differences versus the control group.

creased after treatment with 1,5-AF plus LPS compared with LPS treatment alone (Fig. 4B; $P < 0.01$).

Discussion

The present study revealed that 1,5-AF inhibits LPS-induced NO production in the murine macrophage-like cell line RAW264.7, and protects mice from LPS-induced lung injury by down-regulating the expression of iNOS and up-regulating the production of IL-10.

ROS and NO, an RNS, are produced by phagocytes, such as macrophages, in response to LPS stimulation [19]. The excessive synthesis of NO by iNOS acts as a major macrophage-derived inflammatory mediator and is also involved in the development of inflammatory disease [20]. Our previous study in THP-1 cells showed that 1,5-AF inhibits the formation of ROS because of the presence of enediol forms [9]. Furthermore, other studies have shown that compounds with antioxidant activity such as curcumin [17] and resveratrol [21] also inhibit the production of NO and expression of iNOS. In our present study, 1,5-AF inhibited LPS-induced production of NO and dose-dependently decreased the amount of iNOS protein and its mRNA production in RAW264.7 cells. Thus, these findings suggest that pretreatment with 1,5-AF has an antioxidant effect that may inhibit iNOS expression at the transcriptional and translational levels.

In response to LPS stimulation, the inflammatory cellular infiltrates in the lung predominantly consist of neutrophils and macrophages [22]. In turn, these activated macrophages generate ROS [23] and release many inflammatory mediators, including iNOS [24] and proinflammatory cytokines [25]. This perpetuates a vicious cycle to continue the production of cytotoxic mediators, ultimately leading to profound injury, such as acute lung injury [22]. Moreover, iNOS inhibitors prevent LPS-induced acute respiratory distress syndrome (ARDS) [26]. Our results, which agree with those of previous studies, show that treatment with 1,5-AF significantly suppresses LPS-induced iNOS expression in C57BL/6J mice. This implies that 1,5-AF has an important anti-inflammatory effect on acute lung inflammation.

Increasing the production of the anti-inflammatory cytokine IL-10 could also inhibit proinflammatory mediators such as IL-6 [27] and iNOS [28]. The increased level of IL-10 in the lung of patients with ARDS is associated with improved survival [29] and IL-10-knockout mice show increased iNOS expression and NO production in lung tissue [13]. In the present study, pretreatment with 1,5-AF enhanced the LPS-induced production of the counter-regulatory cytokine IL-10 compared with that with LPS stimulation alone both *in vitro* and *in vivo* and may thus play an inhibitory role in LPS-induced iNOS transcription and translation.

A previous report has revealed that proinflammatory mediators are regulated by the transcription factor NF-κB in LPS-induced lung inflammation [30]. In our study, we found that 1,5-AF slightly suppressed iNOS expression at 3 h (data not shown). However, it did markedly suppress iNOS mRNA and protein expression at 6 h and 12 h, respectively. 1,5-AF is shown to inhibit the translocalization of NF-κB p65 independently of IκBα degradation, and decreased the levels of proinflammatory cytokines such as IL-6,

TNF-α and MCP-1 at 4 h [11]. Furthermore, iNOS expression was dependent on NF-κB activation, thus, suggesting that 1,5-AF may directly inhibit iNOS expression by attenuating NF-κB activation as well as by its antioxidant effects.

The present study indicated that 1,5-AF may inhibit iNOS expression by up-regulation of the anti-inflammatory cytokine IL-10. Since IL-10 has been shown to inhibit the translocalization of NF-κB p65, which was dependent on the degradation of IκBα [31]. The inhibition of iNOS expression (6 h) by 1,5-AF occurred at the same time and consequently increased the expression of IL-10 (6 h). Therefore, these findings suggest that 1,5-AF may directly inhibit iNOS expression and NO production via NF-κB inactivation in the early phase and indirectly via increased IL-10 levels, which may sustain the anti-inflammatory effects of 1,5-AF. Thus, these results raise the possibility that the NF-kB inactivation and increased IL-10 level caused by the action of 1,5-AF may attenuate iNOS expression.

Collectively, our results suggest that 1,5-AF acts as a selective inflammatory inhibitor and this anti-inflammatory effect was augmented by the production of IL-10. In turn, IL-10 inhibited LPS-induced iNOS over-expression in RAW264.7 cells and in the lung tissue of mice. Based on these results, we have clarified the mechanism of 1,5-AF activity, which may be used in the treatment of inflammatory diseases.

Acknowledgments

The authors thank Nobue Uto, Tomomi Morizono, and Tomoka Nagasato for their technical assistance.

This study was supported by Grants-in-Aid 17100007 (to S.T.) and 21390483 (to K.K.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We appreciate NIHON STARCH CO., LTD. for gifting us 1,5-AF.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.06.108.

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Degradation of Vascular Endothelial Thrombomodulin by Arginine- and Lysine-Specific Cysteine Proteases From *Porphyromonas gingivalis*

Megumi Inomata,*† Yuichi Ishihara,† Takashi Matsuyama,‡ Takahisa Imamura,§ Ikuro Maruyama,‖ Toshihide Noguchi,† and Kenji Matsushita*

Background: The endothelial cell surface glycoprotein thrombomodulin (TM) inhibits vascular coagulation and inflammation via regulation of thrombin-mediated activation of protein C. *Porphyromonas gingivalis* is the major periodontopathic bacterium and has been found in vessel walls and atherosclerotic lesions in humans. *P. gingivalis*-derived cysteine proteases (gingipains) are known to enhance inflammatory and coagulant responses of vascular endothelial cells. However, it has not been elucidated whether gingipains affect vascular endothelial TM.

Methods: Purified arginine-specific gingipains (Rgps) and lysine-specific gingipain (Kgp) from *P. gingivalis* were used to investigate the effects of gingipains on recombinant human TM by immunoblot analyses. Flow cytometry and activated protein C assay were carried out to examine the effects of gingipains on vascular endothelial cell surface TM. Immunohistochemistry was performed to investigate TM expression in microvascular endothelia in gingival tissues taken from patients with periodontitis.

Results: Rgps and Kgp cleaved TM in vitro. Endothelial cell surface TM was also degraded by Rgps. Thrombin-mediated activation of protein C was reduced by Rgps through TM inactivation. Gingival microvascular endothelial TM was reduced in patients with periodontitis.

Conclusions: *P. gingivalis* gingipains induced the degradation and inactivation of endothelial TM, which may promote vascular coagulation and inflammation. In addition, in vivo relevance was demonstrated by reduced expression of TM in gingival microvascular endothelia in patients with periodontitis, which may be involved in the pathogenesis of periodontitis. *J Periodontol 2009;80:1511-1517.*

KEY WORDS

Microbiology; periodontitis thrombin; thrombosis.

* Department of Oral Disease Research, National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, Aichi, Japan.
† Department of Periodontology, School of Dentistry, Aichi Gakuin University, Nagoya, Japan.
‡ Department of Periodontology, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan.
§ Division of Molecular Pathology, Department of Neuroscience and Immunology, Kumamoto University Graduate School of Medical Sciences, Kumamoto, Japan.
‖ Department of Laboratory and Molecular Medicine, Faculty of Medicine, Kagoshima University, Kagoshima, Japan.

The major periodontopathic pathogen *Porphyromonas gingivalis* produces cysteine proteases, designated gingipains. Two kinds of arginine residue–specific gingipains, RgpA and RgpB, and another type of lysine residue–specific gingipain, Kgp, have been identified.[1-3] Many studies revealed that gingipains are crucial virulence factors in the development of periodontitis. It was reported that gingipains promote inflammation through the enhancement of vascular permeability by activation of the kallikrein/kinin pathway.[4,5] In addition, gingipains can exert thrombin-like effects in vasculatures, including procoagulant responses and endothelial cell activation through protease-activated receptors.[6,7]

P. gingivalis is often detected in vessel walls and atherosclerotic lesions in humans.[8] Moreover, there is growing evidence that *P. gingivalis* infection is strongly associated with the development of vascular diseases, including coronary heart diseases, stroke, and atherosclerosis.[9-11] Although *P. gingivalis* is known to promote transmigration of leukocytes from blood vessels into inflamed tissue and increase vascular permeability,[10,12-14] these effects of *P. gingivalis* are partly dependent on gingipain-induced activation of vascular endothelial cells to induce an increase in vascular

doi: 10.1902/jop.2009.090114

permeability and adhesion molecule expression.[4,5,15] Therefore, the detailed linkage of proinflammatory and procoagulant responses of vascular endothelial cells to *P. gingivalis*, particularly the responses to gingipains, should be addressed to further understand mechanisms of the development of periodontitis and systemic vascular diseases.

Thrombomodulin (TM) is an endothelial cell surface glycoprotein and contains an extracellular region that harbors a thrombin-binding site.[16] Thrombin binds to TM and activates protein C.[17] Activation of protein C that is dependent on TM causes inhibition of coagulation and inflammatory responses and apoptosis in vascular endothelial cells.[18] Thus, endothelial TM maintains vascular homeostasis through the reduction of thrombotic tendencies and inflammation.

In this study, we investigated the specific involvement of endothelial TM in periodontitis. We investigated whether gingipains directly affect TM in vitro and cell surface TM in human vascular endothelial cells. Also, we examined the expression of TM in the microvascular endothelium in gingival tissues taken from patients with periodontitis.

MATERIALS AND METHODS

Cultivation of Bacteria

The strain of *P. gingivalis* (HG66) was grown in 100 ml broth containing 15.0 g trypticase soy broth,¶ 2.5 g yeast extract, 2.5 mg hemin, 0.25 g cysteine, 0.05 g dithiothreitol, and 0.5 mg menadione, anaerobically, at 37°C for 24 to 30 hours in an atmosphere of 85% N_2, 10% CO_2, 5% H_2. The culture was used to inoculate 2 liters of the same broth, which was then incubated anaerobically at 37°C for ~48 hours until the late stationary phase of bacterium growth.

Purification and Activation of Gingipains

RgpA and Kgp were purified according to the method described by Pike et al.[3] RgpB was purified according to the method described by Potempa et al.[19] The amount of active Rgps or Kgp in each batch of purified proteases was determined by active-site titration with Phe-Pro-Arg-chloromethylketone (FPR-cmk) and benzyloxycarbonyl-L-phenylalanyl-L-lysyl-acyloxyketone (ZFK-ck),# respectively.[20] The same inhibitors were used to obtain inactivated Rgps or Kgp with covalently modified active-site cysteine residues. The concentration of fully activated Rgps or Kgp with cysteine was calculated from the amount of inhibitors needed for complete inactivation of the proteases. Therefore, the concentration of Rgps or Kgp indicated in this study is that of active Rgps or Kgp. To activate Rgps and Kgp, they were diluted with 0.2 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 8.0), 5 mM $CaCl_2$, and 10 mM cysteine and then incubated at 37°C for 10 minutes. The activated gingipains were diluted with serum-free medium for cell culture. To block the enzymatic activity of gingipains, activated gingipains were incubated with FPR-cmk or ZFK-ck for 10 minutes at room temperature before use. The amidolytic activity of purified Rgps or Kgp was determined using benzoyl-L-arginine-*p*-nitroanilide or benzoxycarbonyl-L-lysine-*p*-nitroanilide** as a substrate. The formation of *p*-nitroaniline was monitored spectrophotometrically at 405 nm.

Cell Culture

Human aortic endothelial cells (HAECs)†† were grown as described previously.[21] Cells were used for experiments at passages four to eight.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblot Analysis

A total of 500 nM recombinant human TM (rhTM)‡‡ was incubated at 37°C for 8 hours with RgpA, RgpB, or Kgp at concentrations of 0.1 to 100 nM or for 0 to 8 hours at a concentration of 100 nM. Samples were boiled in a reducing treatment buffer containing 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.05% bromophenol blue and visualized by SDS-PAGE with Coomassie brilliant blue staining. Samples were also separated by SDS-PAGE followed by immunoblot analyses using a monoclonal antibody to TM.§§ Immunoreactive bands were visualized‖ after being treated with a horseradish peroxidase–conjugated antibody to anti-mouse immunoglobulin G (IgG). Cell lysates were obtained by incubating HAECs seeded on 60-mm plates with a buffer consisting of 20 mM Tris-hydrochloride (pH 7.2), 150 mM sodium chloride, 5 mM EDTA, and 1% Triton X-100 in the presence of protease inhibitors at 4°C for 15 minutes followed by clarification by centrifugation at 12,000 × g for 10 minutes. SDS-PAGE and immunoblot analyses were performed as described above. A total of 500 nM recombinant human activated protein C¶¶ was incubated at 37°C for 8 hours with RgpA at concentrations of 1 to 100 nM for an immunoblot analysis using a monoclonal antibody to activated protein C.## Results are representative of three separate experiments.

Flow Cytometry

To assess the surface expression of TM, confluent HAECs were treated with 200 nM RgpA for 1 hour. The cells were removed with phosphate buffered saline containing 20 mM EDTA and fixed with

¶ Difco, Franklin Lakes, NJ.
# Bachem Bioscience, King of Prussia, PA.
** Novabiochem, Darmstadt, Germany.
†† Cambrex, Walkersville, MD.
‡‡ American Diagnostica, Stamford, CT.
§§ Abcam, Tokyo, Japan.
‖ ECL system, Amersham Pharmacia Biotech, Piscataway, NJ.
¶¶ Abcam.
Abcam.

phosphate buffered saline containing 4% paraformaldehyde at 4°C for 1 hour. The cells were incubated at 4°C for 1 hour with anti-TM monoclonal antibody or isotype-matched mouse IgG and then with fluorescein isothiocyanate–conjugated anti-mouse IgG. Samples were analyzed using the flow cytometer.*** Results are representative of three separate experiments.

Protein C Activation Assay

To measure in situ activated protein C–generating capacity, HAECs in 96-well plates were incubated with Dulbecco's modified Eagle's medium in the presence or absence of 1–100 nM RgpA for 8 hours at 37°C, washed with Hanks balanced salt solution (HBSS),††† and incubated with 25 μg/ml recombinant human protein C,‡‡‡ 1 U/ml human thrombin,§§§ 2.5 mM $CaCl_2$, and 1 mg/ml bovine serum albumin in HBSS at 37°C. After 1 hour, the thrombin was neutralized by the addition of 50 mg/ml lepirudin‖‖‖ and incubated with a 3-mM solution of chromogenic substrate for activated protein C S-2366¶¶¶ at 25°C. Hydrolysis of the substrate was determined using a microplate reader.### Results are representative of three separate experiments.

Immunohistochemistry

Tissues were obtained from the healthy gingiva (n = 3; probing depth <3 mm; no bleeding on probing; and no bone loss) and inflamed gingiva (n = 6; probing depth = 3 to 6 mm; bleeding on probing; and bone loss) of nine patients, who had no history or current signs of systemic disease and had received no medication within the prior 6 months, when their teeth were extracted because of deep caries and/or periodontitis. The nine patients (6 females and 3 males; mean age, 46.8 ± 70 years) were enrolled in the study from May 1998 to May 1999. After obtaining informed written consent, the tissues were taken from the marginal gingiva near the extracted socket according to guidelines approved by the Ethics Committee of Kagoshima University Graduate School of Medical and Dental Sciences. At least 10 consecutive sections of three different sites of each gingival tissue were used for immunostaining of TM. Formalin-fixed, decalcified, paraffin-embedded sections were immunostained with an anti-TM monoclonal antibody using a staining system.**** Images were obtained with a microscope.††††

Statistics

All values were evaluated by statistical analysis using the Student-Newman-Keul test. Differences were considered statistically significant at $P < 0.01$.

RESULTS

Rgps and Kgp Degrade TM In Vitro

We first examined whether TM can be a substrate for gingipains in vitro. We found complete digestion of rhTM after incubation with 100 nM purified active

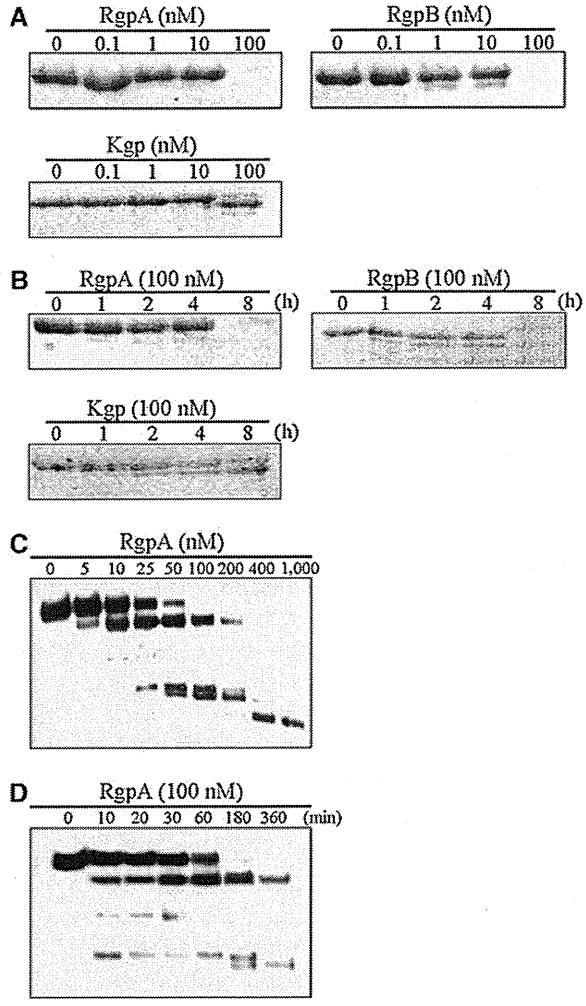

Figure 1.
Rgps and Kgp digested TM in vitro. rhTM was incubated at 37°C for 8 hours with RgpA, RgpB, or Kgp at concentrations of 0.1 to 100 nM ***(A)****, and rhTM was incubated for 0 to 8 hours (h) with 100 nM RgpA, RgpB, or Kgp* ***(B)****. These samples were analyzed by SDS-PAGE. rhTM was treated for 3 hours with RgpA at concentrations of 5 to 1,000 nM* ***(C)****, and rhTM was treated for 0 to 360 minutes (min) with 100 nM RgpA* ***(D)****. These samples were analyzed by SDS-PAGE and immunoblotted with an antibody to TM. The results shown are representative of three separate experiments.*

RgpA or RgpB when assessed by SDS-PAGE (Fig. 1A). Kgp at 100 nM degraded TM, but the effect was very modest. In the case of RgpB, degradation of TM occurred at a concentration <1 nM (Fig. 1A). After treatment with 100 nM RgpB, degradation of TM occurred within 1 hour and continued for ≥8 hours (Fig. 1B).

*** FACSCalibur, Becton Dickinson, Franklin Lakes, NJ.
††† Life Technologies, Gaithersburg, MD.
‡‡‡ Sigma-Aldrich, St. Louis, MO.
§§§ Sigma-Aldrich.
‖‖‖ Peptide Institute, Osaka, Japan.
¶¶¶ Peptide Institute.
Vmax Kinetic Microplate Reader, Molecular Devices, Sunnyvale, CA.
**** sABC kit, Dako, Carpinteria, CA.
†††† Olympus microscope IX71 with DP70 image capture, Olympus, Tokyo, Japan.

RgpA and Kgp at 100 nM required >2 to 4 hours of incubation before obvious degradation was observed (Fig. 1B). Therefore, we focused on Rgps because Rgps digested TM more efficiently than Kgp did. Immunoblot analyses showed that treatment of TM with RgpA led to the formation of low molecular mass fragments in a dose- and time-dependent manner (Figs. 1C and 1D). In addition, immunoblot analyses for TM showed that the fragments were of TM origin, not Rgp origin. It was also confirmed that RgpB could digest rhTM similarly to RgpA (data not shown).

Rgps Cleave Cell Surface TM

We examined whether Rgps affect TM protein in vascular endothelial cells. We previously reported that RgpA at 10 to 1,000 nM did not kill endothelial cells because it could induce exocytosis of endothelial cell–specific components.[6] It is generally believed that dead cells do not release intracellular components. Flow cytometry analysis revealed the presence of a considerable amount of cell surface TM in HAECs (Fig. 2A). After RgpA stimulation, the amount of TM was clearly reduced. In addition, immunoblot analysis showed that RgpA clearly decreased the expression level of TM in HAECs in a dose-dependent manner (Fig. 2B).

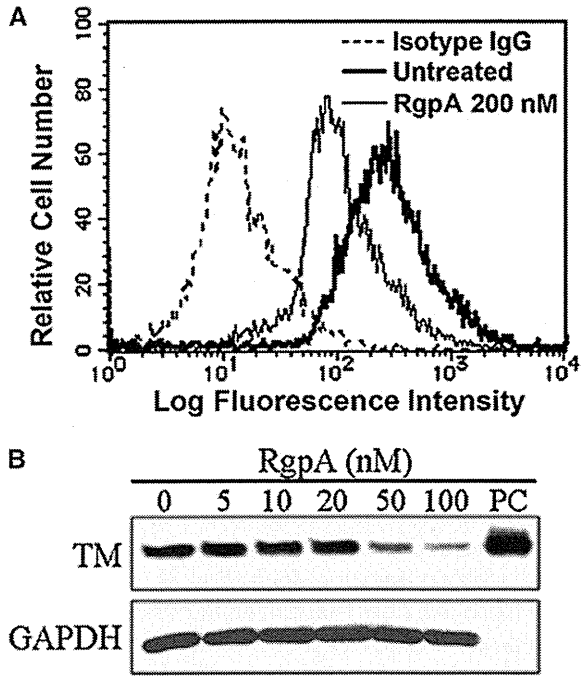

Figure 2.
Rgps degraded TM in cells. ***A)*** *HAECs were treated for 1 hour with 200 nM RgpA, and surface TM was detected by flow cytometry. Thick line = not stimulated; thin line = stimulated with RgpA; dashed line = cells stained with control antibody.* ***B)*** *HAECs were treated at 37°C for 3 hours with RgpA at concentrations of 5 to 100 nM. Immunoblot analysis was performed with an antibody to TM or GAPDH. Representative results of three separate experiments are shown. PC = positive control (rhTM).*

Rgps Inhibit Generation of Activated Protein C by TM

In vascular endothelial cells, thrombin binds to TM and activates protein C. Thrombin treatment led to the activation of protein C in HAECs (Fig. 3A). To clarify whether proteolytic cleavage of TM inhibits TM function, we examined the effect of RgpA on protein C activation. We found that pretreatment of HAECs with RgpA reduced thrombin-mediated activation of protein C in a dose-dependent manner (Fig. 3A). The ability of RgpA to reduce activation of protein C was completely abolished by pretreatment of RgpA with the cysteine protease inhibitor leupeptin (Fig. 3A), indicating that the activity of RgpA depends on its protease activity. We also found that RgpA did not degrade protein C and activated protein C (Fig. 3B and data not shown). These findings suggest that Rgps inhibit TM activity by proteolytic cleavage of TM in vascular endothelial cells.

TM Expression in Gingival Microvascular Endothelia Is Reduced in Patients With Periodontitis

We performed immunohistochemistry for TM in inflamed gingival tissues from human subjects with periodontitis to assess a possible in vivo relevance of endothelial TM to gingipains.

We found that a considerable amount of TM was present in the microvascular endothelia in gingival tissues from healthy subjects (Figs. 4A and 4B). We

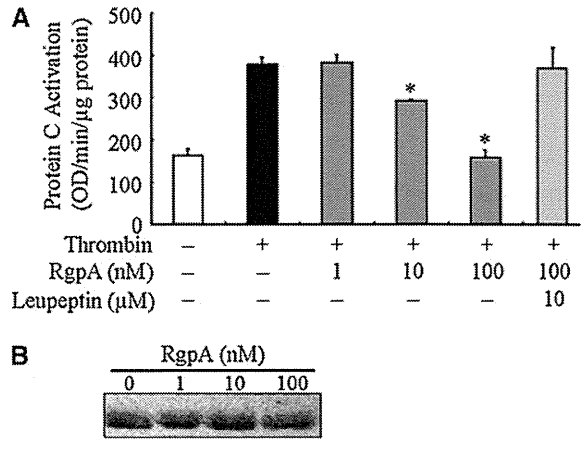

Figure 3.
Rgps inhibit TM activity in vascular endothelial cells. ***A)*** *HAECs were incubated with 1 to 100 nM RgpA for 8 hours in the presence or absence of leupeptin and were washed with HBSS. The cells were treated with 25 μg/ml protein C and 1 U/ml human thrombin. Protein C activation was performed for measurement of TM cofactor activity. Each value is mean ± SD (n = 3).* ***B)*** *Recombinant human activated protein C was incubated at 37°C for 8 hours with RgpA at concentrations of 1 to 100 nM. These samples were analyzed by SDS-PAGE and immunoblotted with an antibody to activated protein C. The results shown are representative of three separate experiments. *Versus cells incubated with thrombin (P <0.01). OD = optical density (405 nm); min = minutes.*

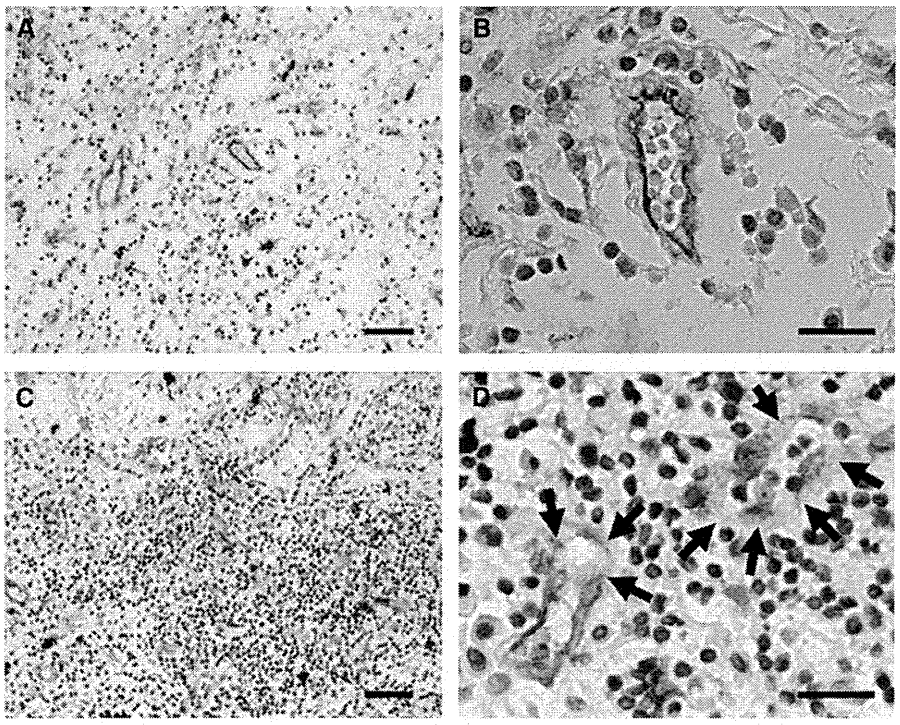

Figure 4.
*Gingival microvascular endothelial TM was reduced in patients with periodontitis. Gingival tissues were sectioned and immunostained with an anti-TM antibody using a staining system.‡‡‡‡ Immunohistochemical staining for TM in healthy **(A** and **B)** or inflamed **(C** and **D)** gingiva from patients with periodontitis. Arrows = decreased staining for TM. Scale bar = 50 μm. All results are representative of three independent experiments.*

also found that the level of TM was clearly lower in the endothelia from subjects with periodontitis than in normal endothelia (Figs. 4C and 4D).

DISCUSSION

The importance of the linkage between endothelial TM and periodontitis has not been clarified. Our study showed that endothelial TM was degraded by Rgps, leading to TM inactivation (Figs. 2 and 3). In addition, the expression level of TM was clearly reduced in the microvascular endothelia in gingival tissues taken from patients with periodontitis (Fig. 4). Although we could not demonstrate that gingipains cause reduction of TM in vivo, given that periodontitis is a disease triggered by bacterial infections, such as *P gingivalis* infection, disruption of TM may be caused by gingipains released from *P. gingivalis* during the development of periodontitis. In addition, because various bacteria other than *P. gingivalis* are involved in the progression of periodontitis, degradation of TM might be induced by gingipains as well as other bacterial proteases and virulence factors.

In vascular endothelial cells, thrombin binds to TM and activates protein C.[17] Activated protein C proteolytically inactivates factors Va and VIIIa, thereby blocking amplification of the coagulation system.[17] It is also known that activated protein C has various other activities, such as suppression of the production of proinflammatory mediators, apoptosis, and E-selectin–dependent leukocyte adhesion in endothelial cells.[18,22-26] TM accelerates protein C activation and directly decreases endothelial cell activation by blocking high-mobility group protein-B1 inflammatory functions and suppressing nuclear factor-kappa B nuclear translocation and the mitogen-activated protein kinase pathways.[27] Thus, TM plays important roles in the maintenance of vascular coagulation and inflammation. Some studies demonstrated that the amount of TM expressed on the endothelial cell surface is decreased upon exposure to the proinflammatory cytokines interleukin (IL)-1 and tumor necrosis factor-alpha (TNF-α).[28,29] Downmodulation of TM expression is associated with a loss in the capacity of endothelial cells to catalyze conversion of protein C to activated protein C, which causes widespread coagulation and inflammation within the microvasculature.[30,31] Furthermore, it has been suggested that diabetes, one of the important risk factors for periodontitis, induces dysfunction of the endothelial TM–protein C system,[32,33] as shown by a decreased level of activated protein C and loss of TM in the endothelium.[34] The perturbation of the TM–protein C system is believed to be a potential mechanism of the progression of diabetic symptoms. Therefore, similar to diabetes, periodontitis may cause dysfunction of the endothelial TM–protein C system triggered by gingipains, which can be an important determinant of the severity of periodontitis. Moreover, such situations could be accelerated by various factors, including IL-1 and TNF-α derived from the host responses in periodontitis.

Gingipains include arginine residue–specific RgpA and RgpB and lysine residue–specific Kgp. In addition, there are two forms of gingipains: vesicle associated and secreted.[35] We tried to purify vesicle-associated RgpA, RgpB, and Kgp because these forms of gingipain exert various pathologic effects through cleavage or degradation of in-host proteins, such as tissue proteins, coagulation factors, and cytokines. It was shown

‡‡‡‡ sABC kit, Dako.

that the enzymatic activity of Rgps is stronger than that of Kgp.[36] In addition, a comparison of the activities of RgpA and RgpB revealed that RgpA exerts more potent activity toward certain types of in-host proteins than does RgpB.[36] Consistent with previous results for Rgps and Kgp, we found that proteolysis of TM by Rgps occurred more strongly than that of TM by Kgp (Fig. 1). In contrast, RgpB digested TM more efficiently than did RgpA (Fig. 1). The different efficiencies of proteolysis may be due to the positions of cleaving sites. It was suggested that RgpA and RgpB possess different substrate specificities.[37] Human TM contains 25 arginine residues, which can be targets for Rgps. RgpA could actually process rhTM, leading to the formation of at least three masses of fragments (Figs. 1C and 1D). In addition, RgpA could also cleave TM in cells (Fig. 2). Because TM also contains two lysine residues, TM can be a major substrate for RgpA, RgpB, and Kgp.

Gingival microvessels are one of the first lines of defense against *P. gingivalis* and the only organs that communicate with the whole body. Although *P. gingivalis* is the most widely studied periodontopathic bacterium contributing to gingival inflammation, many studies[38-41] suggested that *P. gingivalis* could invade endothelial cells and accelerate coagulation and inflammation in aortas, coronary vessels, atherosclerotic plaques, and placenta. Moreover, a recent study[42] showed that prevention of *P. gingivalis* invasion into endothelial cells by antibiotic therapy could reduce the production of proinflammatory cytokines and atherosclerotic plaque development in *P. gingivalis*–infected apolipoprotein $E^{+/-}$ mice. Thus, *P. gingivalis* that has invaded endothelial cells may cause degradation and inactivation of endothelial TM by releasing gingipains, resulting in unbalanced or susceptible procoagulant and immune responses to oral tissues as well as systemic organs.

CONCLUSIONS

Cysteine proteases released from *P. gingivalis* gingipains digest TM, leading to inactivation of its function. Our results may provide new insight into the role of gingipains in the initiation and modulation of vascular coagulant and inflammatory responses. The disruption of TM at sites with high concentrations of gingipains may lead to severe periodontitis or other vascular diseases during the development of *P. gingivalis* infection.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Scientific Research (B: 19390538 to KM), for Exploratory Research (19659494 to KM), and for Aichi Gakuin University High-Tech Research Center Project (to TN), provided by the Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan. The authors report no conflicts of interest related to this study.

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