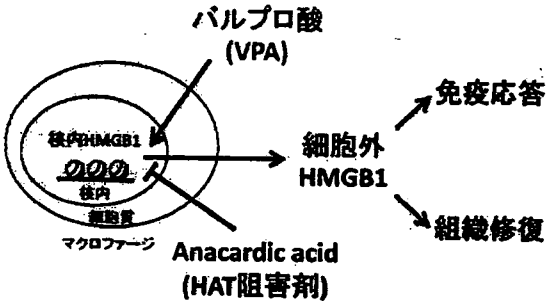

図 1　アセチル化制御薬による HMGB1 の放出制御
HDAC 阻害剤であるバルプロ酸（VPA）により，マクロファージ様細胞から HMGB1 の放出が誘導された。一方，HAT 阻害剤である Anacardic acid（AA）により HMGB1 の放出が抑制された。細胞外に放出された HMGB1 は，免疫応答を創賦する一方，組織修復を促進する可能性がある。

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）。

文　献

1) Li W, Ashok M, Li J, et al.：A major ingredient of green tea rescues mice from lethal sepsis partly by inhibiting HMGB1. PLoS ONE 2 (11)：e1153, 2007
2) Wang H, Bloom O, Zhang M, et al.：HMG-1 as a late mediator of endotoxin lethality in mice. Science 285 (5425)：248-251, 1999
3) Taniguchi N, Yoshida K, Ito T, et al.：Stage-specific secretion of HMGB1 in cartilage regulates endochondral ossification. Mol Cell Biol 27 (16)：5650-5663, 2007
4) Palumbo R, Sampaolesi M, De Marchis F, et al.：Extracellular HMGB1, a signal of tissue damage, induces mesoangioblast migration and proliferation. J Cell Biol 164 (3)：441-449, 2004
5) Lange SS, Mitchell DL, Vasquez KM, et al.：High mobility group protein B1 enhances DNA repair and chromatin modification after DNA damage. Proc Natl Acad Sci U S A 105 (30)：10320-10325, 2008
6) Bonaldi T, Talamo F, Scaffidi P, et al.：Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. EMBO J 22 (20)：5551-5560, 2003
7) Li W, Sama AE, Wang H, et al.：Role of HMGB1 in cardiovascular diseases. Curr Opin Pharmacol 6 (2)：130-135, 2006
8) Adcock IM, Tsaprouni L, Bhavsar P, et al.：Epigenetic regulation of airway inflammation. Curr Opin Immunol 19 (6)：694-700, 2007
9) Duenas-Gonzalez A, Candelaria M, Perez-Plascencia C, et al.：Valproic acid as epigenetic cancer drug：Preclinical, clinical and transcriptional effects on solid tumors. Cancer Treat Rev 34 (3)：206-222, 2008

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

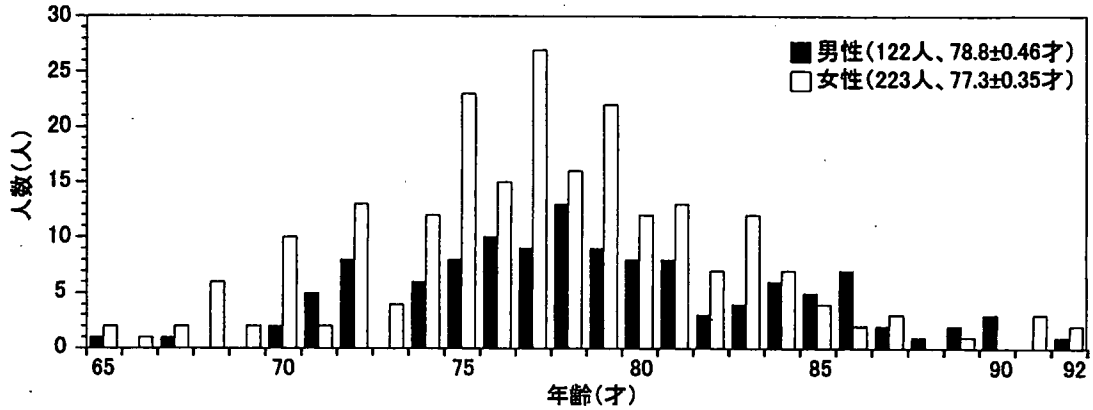

図1．被験者の年齢分布．65歳から92歳までの男性（122人，平均年齢78.8±0.46才），女性（223人，平均年齢77.3±0.35才）について，横軸に年齢を，縦軸にその人数を示した．

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．

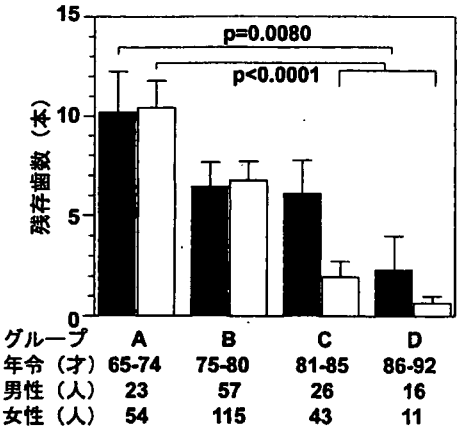

図2．性別高齢者年齢と残存歯数の関係．年齢別に4群（A, B, C, D）に分けて，各群における残存歯数の平均値および標準誤差を示した．A群：65-74才（男性23人, 女性54人），B群：75-80才（男性57人, 女性115人），C群：81-85才（男性26人,女性43人），D群：86-92才（男性16人,女性11人）．A群男性に比べてD群男性は有意に低く（p=0.0080），A群女性（レーン2）に比べてC, D群の女性（レーン8, 10）は共に有意に低かった（p<0.0001）．

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．

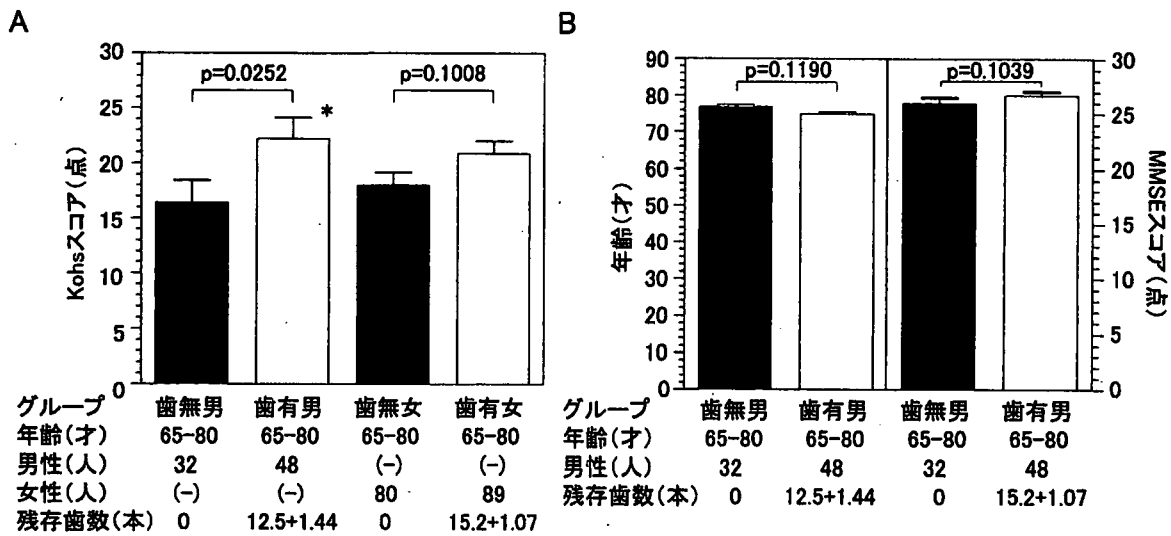

図3．認知機能と歯の有無の関係．65-80歳の男女について簡易版Kohsテスト（パネルA），年齢およびMMSEテスト（パネルB）を行った．歯のない群とある群に分けて，その平均値および標準偏差を表した（歯のない男性32人，歯のある男性48人で残存歯数12.5±1.44本，歯のない女性80人，歯のある女性89人で残存歯数15.2±1.07本）．A）Kohsスコアに関しては男性のみ歯の有無で有意差がみられた（*, p=0.0252）．女性はみられなかった（p=0.1008）．B）年齢およびMMSEスコアに関しては有意差がみられなかった（各々p=0.1190, 0.1039）．

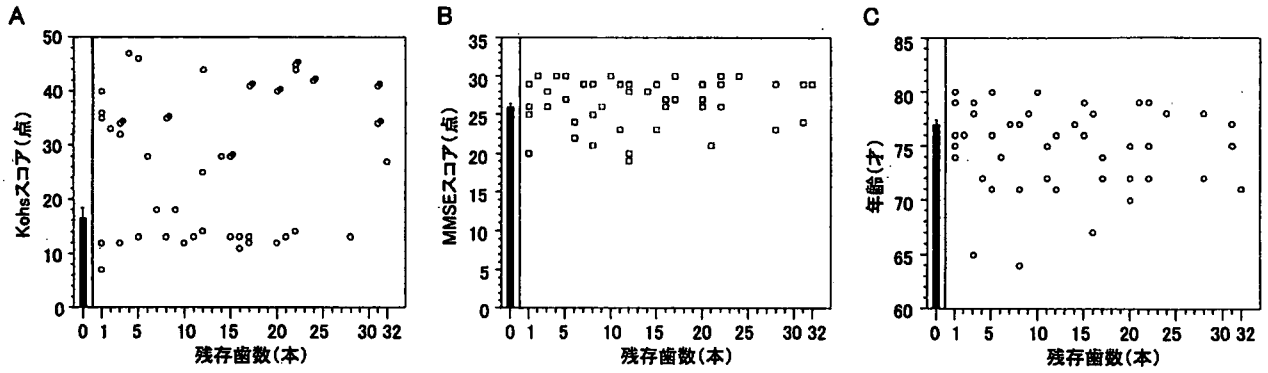

図4．認知機能と残存歯数の関係．65-80歳の男女についてKohsテスト（パネルA），MMSEテスト（パネルB），年齢（パネルC）を行った．残存歯数とKohsスコアをプロットし，両者の相関関係を示した．特に歯のない群の値は棒グラフにすることで歯のある群と差別化した．

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．

参考文献

1）森本 基．8020者データバンクの構築について 口腔保健と全身的な健康状態の関係について（厚生科学研究「口腔保健と全身的な健康状態の関係」運営協議会編），財団法人口腔保健協会，東京，2000：1-11.
2）重富俊雄．口腔機能と老化に関する研究．痴呆の危険因子に関する疫学的検討．口科誌 1998;47:403-407.
3）渡邊 誠，伊藤進太郎．歯の喪失とアルツハイマー型認知症．サイエンスリサーチ 2006；5：36-39.
4）Miura H. Relationship between cognitive function and mastication in elderly females. J OralRehabil 2003; 30: 808-811.
5）Tombaugh TN, McIntyre NJ. The Mini-Mental State Examination: a comprehensive review. J Am Geriatr Soc. 1992; 40: 922-935.
6）Takata Y, Ansai T, Soh I, Sonoki K, Awano S, Hamasaki T, Yoshida A, Ohsumi T, Toyoshima K,

Nishihara T, Takehara T. Cognitive function and number of teeth in a community-dwelling elderly population without dementia. J Oral Rehabil. 2009; 36: 808-813.
7)「香北町健康長寿計画」報告書1990-1995年 香北町, 土佐山田保健所, 高知医科大学老年科, 1996.
8)「香北町健康長寿計画」報告書1996-2001年 香北町, 土佐山田保健所, 高知医科大学老年科, 2002.
9) 松林公蔵, 小澤利男. 後期高齢者の地域における健康管理. Geriatric Medicine 1994; 32: 671-675.
10) 松林公蔵, 小澤利男. 老年者の起居, 動作, 運動機能の客観的評価. Geriatric Medicine 1994; 32: 533-539.
11) 松林公蔵, 小澤利男. 老年者の情緒に関する評価. Geriatric Medicine 1994; 32: 541-546.
12) 松林公蔵, 奥宮清人, 河本昭子, 木村茂昭, 和田知子, 藤澤道子, 土居義典, 島田和幸, 小澤利男. 地域在住者の自立度に関する経時的変化. 日本老年医学会雑誌 1994；31：214-220.
13) Shimada K, Ozawa T, Matsubayashi K. Dependency of the aged in the community. Lancet 1993; 342: 185.
14) Matsubayashi K, Okumiya K, Wada T, Doi Y, Ozawa T. Secular improvement in self-care independence of old people living in community in Kahoku, Japan. Lancet 1996; 347: 60.
15) Matsubayashi K, Okumiya K, Wada T, Osaki Y, Fujisawa M, Doi Y, Ozawa T. Postural dysregulation in systolic blood pressure is associated with worsened scoring on neurobehavioral function tests and leukoaraiosis in the older elderly living in a community. Stroke 1997; 28: 2169-2173.
16) Matsubayashi K, Okumiya K, Wada T, Doi Y, Ozawa T. High blood-pressure control in Japanese hypertensive population. The Lancet 1997; 350: 290-291.
17) Matsubayashi K. Sex and examination results. Lancet 1997; 350: 1711.
18) Matsubayashi K, Okumiya K, Osaki Y, Fujisawa M, Doi Y. Quality of life of old people living in the community. Lancet 1997; 350: 1521-1522.
19) Matsubayashi K, Okumiya K, Nakamura T, Fujisawa M, Osaki Y. Global burden of disease. Lancet 1997; 350: 144.
20) Matsubayashi K, Okumiya K, Osaki Y, Fujisawa M, Doi Y. Frailty in elderly Japanese. Lancet 1999; 353: 1445.
21) Okumiya K, Matsubayashi K, Nakamura T, Fujisawa M, Osaki Y, Doi Y, Ozawa T. The timed "Up & Go" test and manual button score are useful predictors of functional decline in basic and instrumental ADL in community-dwelling older people. J Am Geriatr Soc 1999; 47: 497-498.
22) 西永正典. 総合機能評価（CGA）の臨床応用とその意義. 日老医誌 2000；37：859-865.
23) 西永正典. CGAツールとその特徴. 老年医学 2001；39：1493-1499.
24) 厚生省健康政策局歯科衛生課編. 平成5年歯科疾患実態調査報告. 東京：口腔保健協会；1995.
25) Kohs SC. The Block-Design Tests. J Ex Psychol. 1920; 3: 357-376.
26) Grabe HJ, Schwahn C, Völzke H, Spitzer C, Freyberger HJ, John U, Mundt T, Biffar R, Kocher T. Tooth loss and cognitive impairment. J Clin Periodontol. 2009; 36: 550-557.
27) Avlund K, Holm-Pedersen P, Morse DE, Viitanen M, Winblad B. Tooth loss and caries prevalence in very old Swedish people: the relationship to cognitive function and functional ability. Gerodontology. 2004; 21: 17-26.

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

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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.

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
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

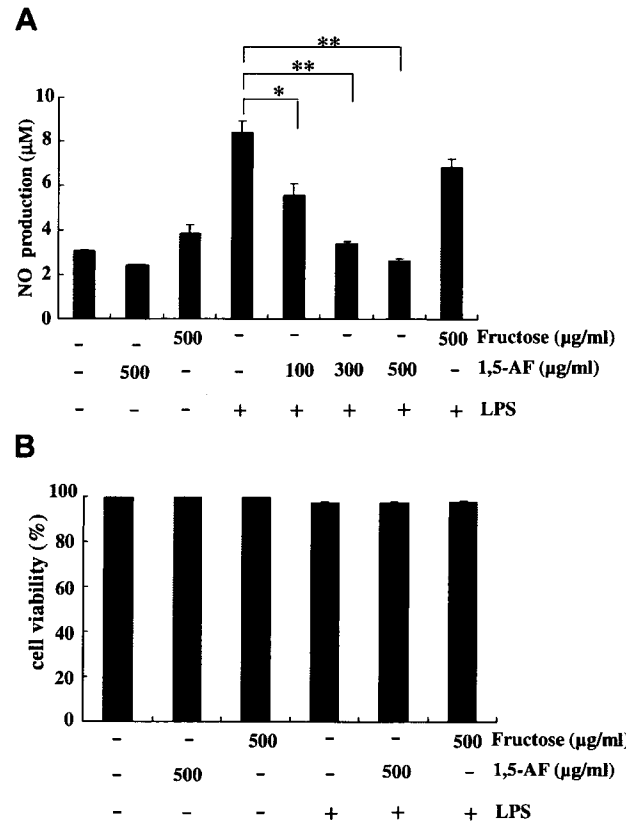

Fig. 1. 1,5-AF inhibits LPS-induced NO production in RAW264.7 cells. Cells were pretreated for 2 h with the indicated concentration of 1,5-AF and were then stimulated with LPS (500 ng/ml) for 18 h. (A) The culture media were collected and assayed for nitrite production. (B) Cells were collected and assayed 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.

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-

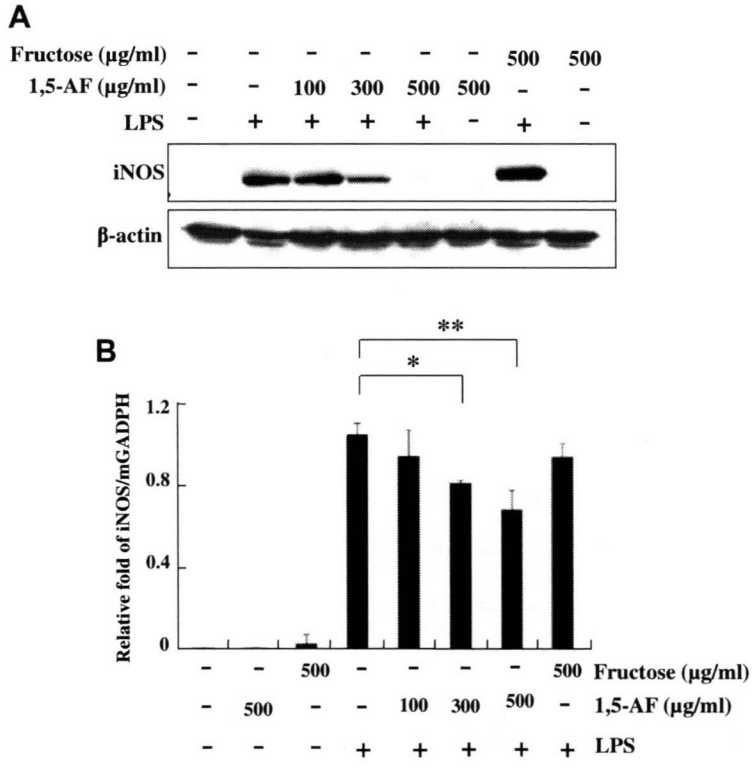

Fig. 2. 1,5-AF inhibits LPS-induced iNOS protein levels and mRNA expression in RAW264.7 cells. Cells were pretreated for 2 h with the indicated concentration of 1,5-AF and were then stimulated with LPS (500 ng/ml). (A) Whole-cell lysates were prepared after 12 h of stimulation, and were analyzed for 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.

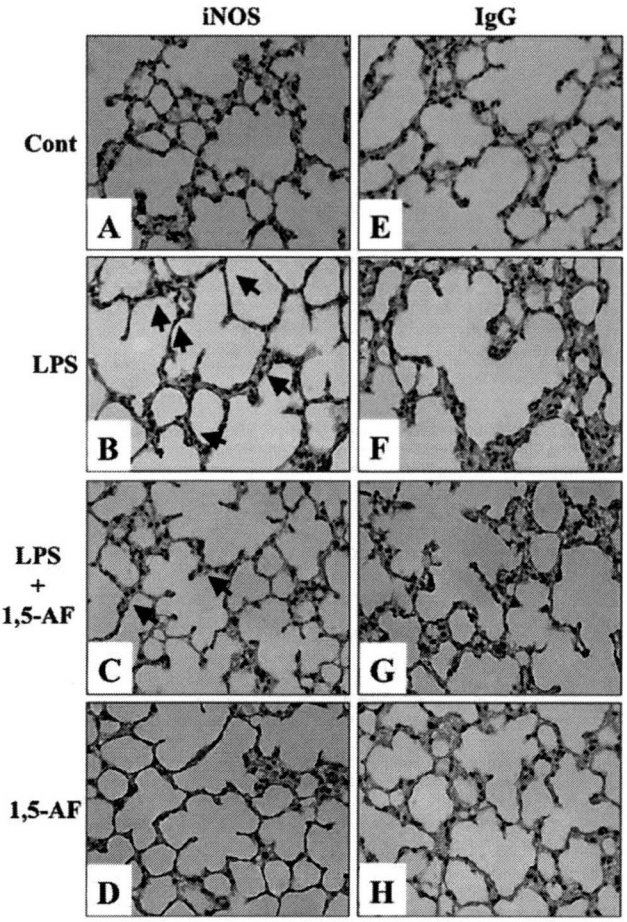

Fig. 3. 1,5-AF inhibits LPS-induced iNOS expression in the lung tissue of C57BL/6J mice. Seven-week-old male mice were challenged for 4 h with either saline alone (A) or LPS (B) immediately after 1,5-AF (38.5 mg/kg) (C) or saline treatment and 1,5-AF alone (D). The lung tissues were stained with iNOS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

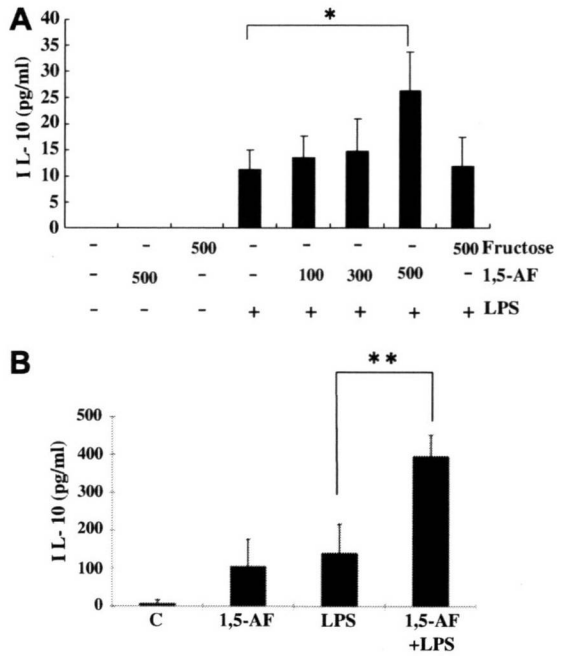

Fig. 4. 1,5-AF upregulates IL-10 production in LPS-stimulated RAW264.7 cells and mice serum. (A) Cells were pretreated for 2 h with the indicated concentration of 1,5-AF and were then stimulated with LPS (500 ng/ml) for 6 h. The concentration of IL-10 released into the supernatant was measured by ELISA. 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.

References

[1] M. Li, D.F. Carpio, Y. Zheng, P. Bruzzo, V. Singh, F. Ouaaz, R.M. Medzhitov, A.A. Beg, An essential role of the NF-kappa B/Toll-like receptor pathway in induction of inflammatory and tissue-repair gene expression by necrotic cells, J. Immunol. 166 (2001) 7128–7135.
[2] A. Petros, D. Bennett, P. Vallance, Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock, Lancet 338 (1991) 1557–1558.
[3] Y. Vodovotz, M.S. Lucia, K.C. Flanders, L. Chesler, Q.W. Xie, T.W. Smith, J. Weidner, R. Mumford, R. Webber, C. Nathan, A.B. Roberts, C.F. Lippa, M.B. Sporn, Inducible nitric oxide synthase in tangle-bearing neurons of patients with Alzheimer's disease, J. Exp. Med. 184 (1996) 1425–1433.
[4] S. Yu, K. Bojsen, B. Svensson, J. Marcussen, Alpha-1, 4-glucan lyases producing 1, 5-anhydro-D-fructose from starch and glycogen have sequence similarity to alpha-glucosidases, Biochim. Biophys. Acta 1433 (1999) 1–15.
[5] M.-A. Baute, R. Baute, G. Deffieux, Fungal enzymic activity degrading 1, 4-[alpha]-D-glucans to 1, 5-D-anhydrofructose, Phytochemistry 27 (1988) 3401–3403.
[6] A. Broberg, L. Kenne, M. Pedersen, Analysis of 1,5-anhydro-D-fructose, microthecin, and 4-deoxy-glycero-hexo-2,3-diulose in algae using gas chromatography–mass spectrometry in selected ion monitoring mode, Anal. Biochem. 268 (1999) 35–42.
[7] Y. Shiga, S. Kametani, T. Kadokura, H. Akanuma, 1, 5-Anhydroglucitol promotes glycogenolysis in *Escherichia coli*, J. Biochem. 125 (1999) 166–172.
[8] S. Kametani, Y. Shiga, H. Akanuma, Hepatic production of 1,5-anhydrofructose and 1,5-anhydroglucitol in rat by the third glycogenolytic pathway, Eur. J. Biochem. 242 (1996) 832–838.
[9] K. Yamaji, K.P. Sarker, I. Maruyama, S. Hizukuri, Antioxidant effects of 1,5-anhydro-D-fructose a new natural sugar in vitro, Planta Med. 68 (2002) 16–19.
[10] S. Yu, R. Fiskesund, The anhydrofructose pathway and its possible role in stress response and signaling, Biochim. Biophys. Acta 1760 (2006) 1314–1322.
[11] X. Meng, K. Kawahara, Y. Nawa, N. Miura, B. Shrestha, S. Tancharoen, H. Sameshima, T. Hashiguchi, I. Maruyama, 1,5-Anhydro-D-fructose attenuates lipopolysaccharide-induced cytokine release via suppression of NF-kappaB p65 phosphorylation, Biochem. Biophys. Res. Commun. 380 (2009) 343–348.
[12] C.W. Chow, M.T. Herrera Abreu, T. Suzuki, G.P. Downey, Oxidative stress and acute lung injury, Am. J. Respir. Cell Mol. Biol. 29 (2003) 427–431.
[13] S. Cuzzocrea, E. Mazzon, L. Dugo, I. Serraino, R. Di Paola, T. Genovese, A. De Sarro, A.P. Caputi, Absence of endogenous interleukin-10 enhances the evolution of acute lung injury, Eur. Cytokine Network 13 (2002) 285–297.
[14] T. Uto, M. Fujii, D.X. Hou, 6-(Methylsulfinyl)hexyl isothiocyanate suppresses inducible nitric oxide synthase expression through the inhibition of Janus kinase 2-mediated JNK pathway in lipopolysaccharide-activated murine macrophages, Biochem. Pharmacol. 70 (2005) 1211–1221.
[15] T.P. Misko, R.J. Schilling, D. Salvemini, W.M. Moore, M.G. Currie, A fluorometric assay for the measurement of nitrite in biological samples, Anal. Biochem. 214 (1993) 11–16.
[16] K. Kawahara, K.K. Biswas, M. Unoshima, T. Ito, K. Kikuchi, Y. Morimoto, M. Iwata, S. Tancharoen, Y. Oyama, K. Takenouchi, Y. Nawa, N. Arimura, M.X. Jie, B. Shrestha, N. Miura, T. Shimizu, K. Mera, S. Arimura, N. Taniguchi, H. Iwasaka, S. Takao, T. Hashiguchi, I. Maruyama, C-reactive protein induces high-mobility group box-1 protein release through activation of p38MAPK in macrophage RAW264.7 cells, Cardiovasc. Pathol. 17 (2008) 129–138.
[17] J.A. Epstein, Currying favor for the heart, J. Clin. Invest. 118 (2008) 850–852.
[18] C.L. Speyer, T.A. Neff, R.L. Warner, R.F. Guo, J.V. Sarma, N.C. Riedemann, M.E. Murphy, H.S. Murphy, P.A. Ward, Regulatory effects of iNOS on acute lung inflammatory responses in mice, Am. J. Pathol. 163 (2003) 2319–2328.
[19] A.H. Ding, C.F. Nathan, D.J. Stuehr, Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production, J. Immunol. 141 (1988) 2407–2412.
[20] A.K. Nussler, T.R. Billiar, Inflammation, immunoregulation, and inducible nitric oxide synthase, J. Leukoc. Biol. 54 (1993) 171–178.
[21] N. Kawada, S. Seki, M. Inoue, T. Kuroki, Effect of antioxidants, Resveratrol, quercetin, and *N*-acetylcysteine, on the functions of cultured rat hepatic stellate cells and Kupffer cells, Hepatology 27 (1998) 1265–1274.
[22] W. MacNee, Oxidative stress and lung inflammation in airways disease, Eur. J. Pharmacol. 429 (2001) 195–207.
[23] J.T. Myers, J.A. Swanson, Calcium spikes in activated macrophages during Fcgamma receptor-mediated phagocytosis, J. Leukoc. Biol. 72 (2002) 677–684.
[24] B. Gaston, J.M. Drazen, J. Loscalzo, J.S. Stamler, The biology of nitrogen oxides in the airways, Am. J. Respir. Crit. Care Med. 149 (1994) 538–551.
[25] W.W. Lin, M. Karin, A cytokine-mediated link between innate immunity, inflammation, and cancer, J. Clin. Invest. 117 (2007) 1175–1183.
[26] M. Numata, S. Suzuki, N. Miyazawa, A. Miyashita, Y. Nagashima, S. Inoue, T. Kaneko, T. Okubo, Inhibition of inducible nitric oxide synthase prevents LPS-induced acute lung injury in dogs, J. Immunol. 160 (1998) 3031–3037.
[27] J.R. Heyen, S. Ye, B.N. Finck, R.W. Johnson, Interleukin (IL)-10 inhibits IL-6 production in microglia by preventing activation of NF-kappaB, Brain Res. Mol. Brain Res. 77 (2000) 138–147.
[28] D. Ogando, M. Cella, M.L. Ribeiro, C. Weissmann, J. Aisemberg, A. Franchi, IL-10 inhibits nitric oxide synthesis in murine uterus, Neuroimmunomodulation 11 (2004) 127–132.
[29] S.C. Donnelly, R.M. Strieter, P.T. Reid, S.L. Kunkel, M.D. Burdick, I. Armstrong, A. Mackenzie, C. Haslett, The association between mortality rates and decreased concentrations of interleukin-10 and interleukin-1 receptor antagonist in the lung fluids of patients with the adult respiratory distress syndrome, Ann. Intern. Med. 125 (1996) 191–196.
[30] H.S. Lee, C. Moon, H.W. Lee, E.M. Park, M.S. Cho, J.L. Kang, Src tyrosine kinases mediate activations of NF-kappaB and integrin signal during lipopolysaccharide-induced acute lung injury, J. Immunol. 179 (2007) 7001–7011.
[31] R. Al-Ashy, I. Chakroun, M.E. El-Sabban, F.R. Homaidan, The role of NF-kappaB in mediating the anti-inflammatory effects of IL-10 in intestinal epithelial cells, Cytokine 36 (2006) 1–8.

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 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).

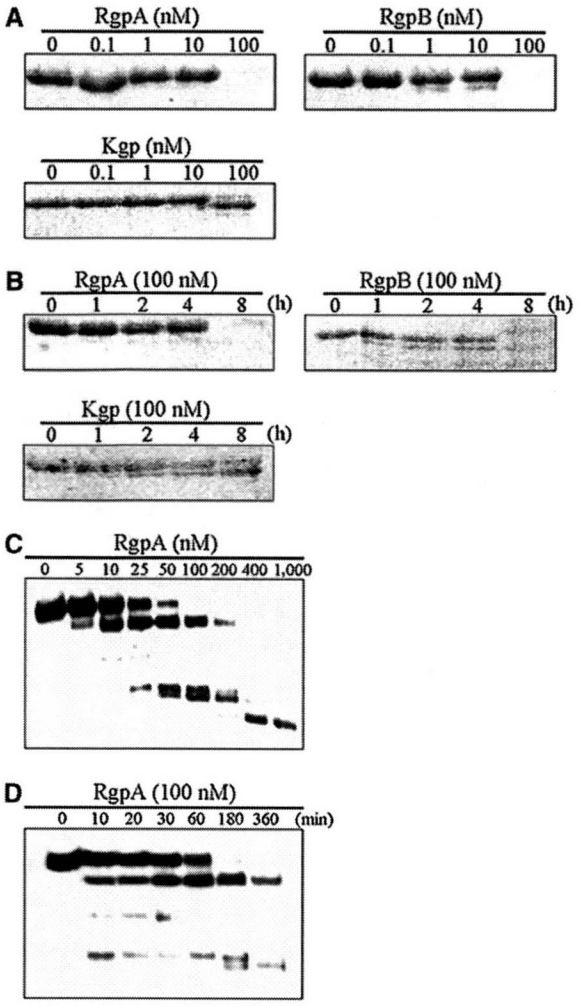

Figure 1.

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.*

*** 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).

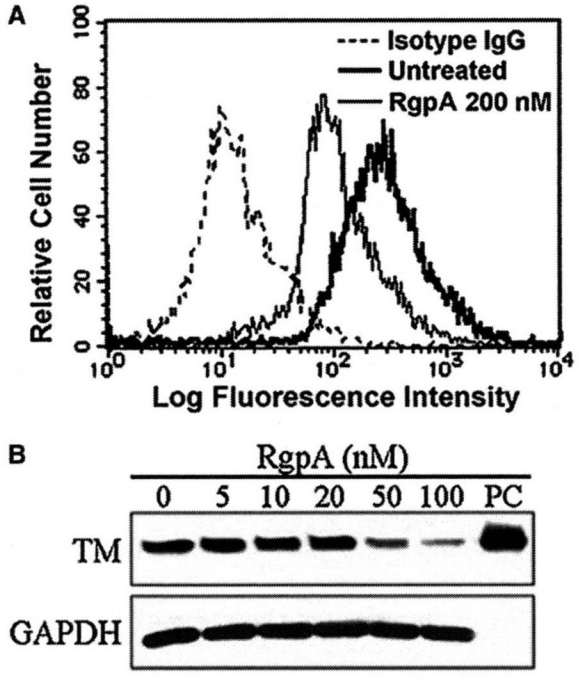

Figure 2.
Rgps degraded TM in cells. ***A)*** *HAECs were treated for 1 hour with 200 nM RgpA, and surface TM was detected by flow cytometry. Thick line = not stimulated; thin line = stimulated with RgpA; dashed line = cells stained with control antibody.* ***B)*** *HAECs were treated at 37°C for 3 hours 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

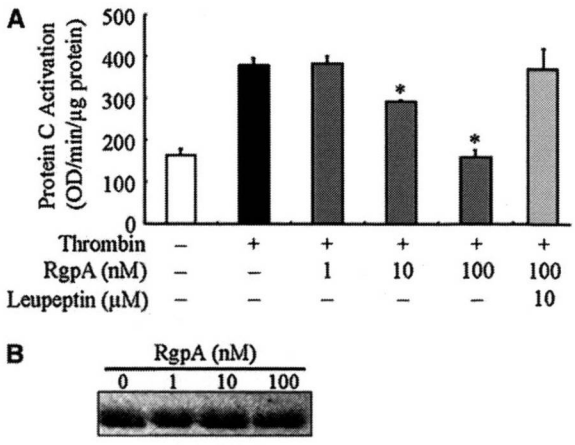

Figure 3.
Rgps inhibit TM activity in vascular endothelial cells. ***A)*** *HAECs were incubated with 1 to 100 nM RgpA for 8 hours in the presence or absence of leupeptin and were washed with HBSS. The cells were treated with 25 μg/ml protein C and 1 U/ml human thrombin. Protein C activation was performed 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.*

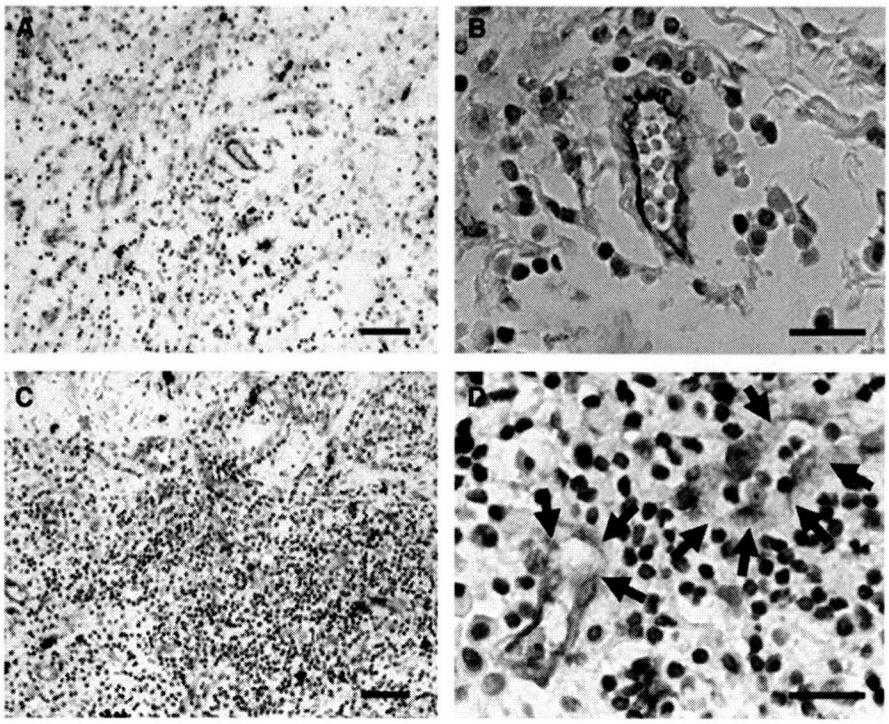

Figure 4.
*Gingival microvascular endothelial TM was reduced in patients with periodontitis. Gingival tissues were sectioned and immunostained with an anti-TM antibody using a staining system.‡‡‡‡ Immunohistochemical staining for TM in healthy **(A** and **B)** or inflamed **(C** and **D)** gingiva from 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.

REFERENCES

1. Chen Z, Potempa J, Polanowski A, Wikstrom M, Travis J. Purification and characterization of a 50-kDa cysteine proteinase (gingipain) from *Porphyromonas gingivalis*. *J Biol Chem* 1992;267:18896-18901.
2. Kadowaki T, Yoneda M, Okamoto K, Maeda K, Yamamoto K. Purification and characterization of a novel arginine-specific cysteine proteinase (argingipain) involved in the pathogenesis of periodontal disease from the culture supernatant of *Porphyromonas gingivalis*. *J Biol Chem* 1994;269:21371-21378.
3. Pike R, McGraw W, Potempa J, Travis J. Lysine- and arginine-specific proteinases from *Porphyromonas gingivalis*. Isolation, characterization, and evidence for the existence of complexes with hemagglutinins. *J Biol Chem* 1994;269:406-411.
4. Imamura T, Pike RN, Potempa J, Travis J. Pathogenesis of periodontitis: A major arginine-specific cysteine proteinase from *Porphyromonas gingivalis* induces vascular permeability enhancement through activation of the kallikrein/kinin pathway. *J Clin Invest* 1994;94:361-367.
5. Imamura T, Potempa J, Pike RN, Travis J. Dependence of vascular permeability enhancement on cysteine proteinases in vesicles of *Porphyromonas gingivalis*. *Infect Immun* 1995;63:1999-2003.
6. Inomata M, Into T, Ishihara Y, Nakashima M, Noguchi T, Matsushita K. Arginine-specific gingipain A from *Porphyromonas gingivalis* induces Weibel-Palade body exocytosis and enhanced activation of vascular endothelial cells through protease-activated receptors. *Microbes Infect* 2007;9:1500-1506.
7. Lourbakos A, Yuan YP, Jenkins AL, et al. Activation of protease-activated receptors by gingipains from *Porphyromonas gingivalis* leads to platelet aggregation: A new trait in microbial pathogenicity. *Blood* 2001;97:3790-3797.
8. Desvarieux M, Demmer RT, Rundek T, et al. Periodontal microbiota and carotid intima-media thickness: The Oral Infections and Vascular Disease Epidemiology Study (INVEST). *Circulation* 2005;111:576-582.
9. Beck JD, Elter JR, Heiss G, Couper D, Mauriello SM, Offenbacher S. Relationship of periodontal disease to carotid artery intima-media wall thickness: The atherosclerosis risk in communities (ARIC) study. *Arterioscler Thromb Vasc Biol* 2001;21:1816-1822.
10. Genco RJ, Trevisan M, Wu T, Beck JD. Periodontal disease and risk of coronary heart disease. *JAMA* 2001;285:40-41.
11. Mattila KJ, Valtonen VV, Nieminen MS, Asikainen S. Role of infection as a risk factor for atherosclerosis, myocardial infarction, and stroke. *Clin Infect Dis* 1998;26:719-734.
12. Cutler CW, Kalmar JR, Genco CA. Pathogenic strategies of the oral anaerobe, *Porphyromonas gingivalis*. *Trends Microbiol* 1995;3:45-51.
13. Genco RJ. Host responses in periodontal diseases: Current concepts. *J Periodontol* 1992;63:338-355.
14. Saglie FR, Marfany A, Camargo P. Intragingival occurrence of *Actinobacillus actinomycetemcomitans* and *Bacteroides gingivalis* in active destructive periodontal lesions. *J Periodontol* 1988;59:259-265.

15. Imamura T, Potempa J, Pike RN, Moore JN, Barton MH, Travis J. Effect of free and vesicle-bound cysteine proteinases of *Porphyromonas gingivalis* on plasma clot formation: Implications for bleeding tendency at periodontitis sites. *Infect Immun* 1995;63:4877-4882.
16. Maruyama I, Bell CE, Majerus PW. Thrombomodulin is found on endothelium of arteries, veins, capillaries, and lymphatics, and on syncytiotrophoblast of human placenta. *J Cell Biol* 1985;101:363-371.
17. Esmon CT. The roles of protein C and thrombomodulin in the regulation of blood coagulation. *J Biol Chem* 1989;264:4743-4746.
18. Esmon CT. Inflammation and the activated protein C anticoagulant pathway. *Semin Thromb Hemost* 2006; 32(Suppl. 1):49-60.
19. Potempa J, Mikolajczyk-Pawlinska J, Brassell D, et al. Comparative properties of two cysteine proteinases (gingipains R), the products of two related but individual genes of *Porphyromonas gingivalis*. *J Biol Chem* 1998;273:21648-21657.
20. Potempa J, Pike R, Travis J. Titration and mapping of the active site of cysteine proteinases from *Porphyromonas gingivalis* (gingipains) using peptidyl chloromethanes. *Biol Chem* 1997;378:223-230.
21. Into T, Kanno Y, Dohkan J, et al. Pathogen recognition by Toll-like receptor 2 activates Weibel-Palade body exocytosis in human aortic endothelial cells. *J Biol Chem* 2007;282:8134-8141.
22. Esmon CT. The protein C pathway. *Chest* 2003;124: 26S-32S.
23. Esmon CT. Interactions between the innate immune and blood coagulation systems. *Trends Immunol* 2004;25:536-542.
24. Rezende SM, Simmonds RE, Lane DA. Coagulation, inflammation, and apoptosis: Different roles for protein S and the protein S-C4b binding protein complex. *Blood* 2004;103:1192-1201.
25. Riewald M, Petrovan RJ, Donner A, Mueller BM, Ruf W. Activation of endothelial cell protease activated receptor 1 by the protein C pathway. *Science* 2002;296: 1880-1882.
26. Weiler H, Isermann BH. Thrombomodulin. *J Thromb Haemost* 2003;1:1515-1524.
27. Van de Wouwer M, Conway EM. Novel functions of thrombomodulin in inflammation. *Crit Care Med* 2004;32:S254-S261.
28. Nawroth PP, Bank I, Handley D, Cassimeris J, Chess L, Stern D. Tumor necrosis factor/cachectin interacts with endothelial cell receptors to induce release of interleukin 1. *J Exp Med* 1986;163:1363-1375.
29. Nawroth PP, Handley DA, Esmon CT, Stern DM. Interleukin 1 induces endothelial cell procoagulant while suppressing cell-surface anticoagulant activity. *Proc Natl Acad Sci USA* 1986;83:3460-3464.
30. Moore KL, Esmon CT, Esmon NL. Tumor necrosis factor leads to the internalization and degradation of thrombomodulin from the surface of bovine aortic endothelial cells in culture. *Blood* 1989;73:159-165.
31. Pober JS, Cotran RS. The role of endothelial cells in inflammation. *Transplantation* 1990;50:537-544.
32. Borcea V, Morcos M, Isermann B, et al. Influence of ramipril on the course of plasma thrombomodulin in patients with diabetes mellitus. *Vasa* 1999;28:172-180.
33. Fujiwara Y, Tagami S, Kawakami Y. Circulating thrombomodulin and hematological alterations in type 2 diabetic patients with retinopathy. *J Atheroscler Thromb* 1998;5:21-28.
34. Isermann B, Vinnikov IA, Madhusudhan T, et al. Activated protein C protects against diabetic nephropathy by inhibiting endothelial and podocyte apoptosis. *Nat Med* 2007;13:1349-1358.
35. Potempa J, Sroka A, Imamura T, Travis J. Gingipains, the major cysteine proteinases and virulence factors of *Porphyromonas gingivalis*: Structure, function and assembly of multidomain protein complexes. *Curr Protein Pept Sci* 2003;4:397-407.
36. Mezyk-Kopec R, Bzowska M, Potempa J, et al. Inactivation of membrane tumor necrosis factor alpha by gingipains from *Porphyromonas gingivalis*. *Infect Immun* 2005;73:1506-1514.
37. Ally N, Whisstock JC, Sieprawska-Lupa M, et al. Characterization of the specificity of arginine-specific gingipains from *Porphyromonas gingivalis* reveals active site differences between different forms of the enzymes. *Biochemistry* 2003;42:11693-11700.
38. Bedi GS, Williams T. Purification and characterization of a collagen-degrading protease from *Porphyromonas gingivalis*. *J Biol Chem* 1994;269:599-606.
39. Cavrini F, Sambri V, Moter A, et al. Molecular detection of *Treponema denticola* and *Porphyromonas gingivalis* in carotid and aortic atheromatous plaques by FISH: Report of two cases. *J Med Microbiol* 2005; 54:93-96.
40. Deshpande RG, Khan MB, Genco CA. Invasion of aortic and heart endothelial cells by *Porphyromonas gingivalis*. *Infect Immun* 1998;66:5337-5343.
41. Yumoto H, Chou HH, Takahashi Y, Davey M, Gibson FC 3rd, Genco CA. Sensitization of human aortic endothelial cells to lipopolysaccharide via regulation of Toll-like receptor 4 by bacterial fimbria-dependent invasion. *Infect Immun* 2005;73:8050-8059.
42. Amar S, Wu SC, Madan M. Is *Porphyromonas gingivalis* cell invasion required for atherogenesis? Pharmacotherapeutic implications. *J Immunol* 2009;182: 1584-1592.

Correspondence: Dr. Kenji Matsushita, Department of Oral Disease Research, National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, 36-3 Gengo, Obu, Aichi 474-8522, Japan. Fax: 81-562-46-8479; e-mail: kmatsu30@nils.go.jp.

Submitted February 24, 2009; accepted for publication April 13, 2009.

The American Journal of Pathology, Vol. 175, No. 5, November 2009

DOI: 10.2353/ajpath.2009.080705

Cell Injury, Repair, Aging and Apoptosis

Matrix Metalloproteinase-3 Accelerates Wound Healing following Dental Pulp Injury

Li Zheng,* Kazuharu Amano,† Koichiro Iohara,* Masataka Ito,‡ Kiyomi Imabayashi,* Takeshi Into,* Kenji Matsushita,* Hiroshi Nakamura,† and Misako Nakashima*

From the Department of Oral Disease Research, National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, Aichi; the Department of Endodontics,† School of Dentistry, Aichi-Gakuin University, Nagoya, and the Department of Developmental Anatomy and Regenerative Medicine,‡ National Defense Medical College, Saitama, Japan*

Matrix metalloproteinases (MMPs) are implicated in a wide range of physiological and pathological processes, including morphogenesis, wound healing, angiogenesis, inflammation, and cancer. Angiogenesis is essential for reparative dentin formation during pulp wound healing. The mechanism of angiogenesis, however, still remains unclear. We hypothesized that certain MMPs expressed during pulp wound healing may support recovery processes. To address this issue, a rat pulp injury model was established to investigate expression of MMPs during wound healing. Real-time RT-PCR analysis showed that expression MMP-3 and MMP-9 (albeit lower extent) was up-regulated at 24 and 12 hours after pulp injury, respectively, whereas expression of MMP-2 and MMP-14 was not changed. MMP-3 mRNA and protein were localized in endothelial cells and/or endothelial progenitor cells in injured pulp *in vivo*. In addition, MMP-3 enhanced proliferation, migration, and survival of human umbilical vein endothelial cells *in vitro*. Furthermore, the topical application of MMP-3 protein on the rat-injured pulp tissue *in vivo* induced angiogenesis and reparative dentin formation at significantly higher levels compared with controls at 24 and 72 hours after treatment, respectively. Inhibition of endogenous MMP-3 by *N*-Isobutyl-*N*-(4-methoxyphenylsulfonyl)-glycylhydroxamic acid resulted in untoward wound healing. These results provide suggestive evidence that MMP-3 released from endothelial cells and/or endothelial progenitor cells in injured pulp plays critical roles in angiogenesis and pulp wound healing. ***(Am J Pathol 2009, 175:1905–1914; DOI: 10.2353/ajpath.2009.080705)***

Matrix metalloproteinases (MMPs) comprise a zinc-dependent endopeptidase family of at least 24 mammalian members.[1–4] MMPs act on a variety of substrates, including extracellular matrix (ECM) proteins, proteinases, and their inhibitors, to activate latent growth factors, cytokines, chemokines, receptors, and adhesion molecules or to alter protein function, such as shedding of cell surface proteins.[5,6] Thus, MMPs have been implicated in a wide range of physiological and pathological processes, including embryonic development, wound healing, tissue remodeling, angiogenesis, morphogenesis, inflammation, and cancer.[7–9]

Wound healing is a multifactorial process involving the migration, proliferation, and differentiation into several cell populations with subsequent formation of extracellular matrix. MMPs may influence cell migration through extracellular matrix degradation or by altering cellular adhesive properties. MMPs may also stimulate proliferation and/or antiapoptosis by altering the extracellular matrix microenvironment. They further modulate the activity of growth factors and receptors.[10,11]

On the basis of their ability, MMPs participate in wound healing and angiogenesis.[4] Several different MMPs play a beneficial role in wound-healing models. During the wound-healing process of skin, CXC chemokine receptor (CXCR) 4^+ cells migrating around vessels release

Supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan (no. 19659499), and Aichi-Gakuin University "High-Tech Research Center" Project for Private Universities: matching fund subsidy from Ministry of Education, Culture, Sports, Science and Technology, Japan, 2003–2007.

L.Z. and K.A. contributed equally to this work.

Accepted for publication July 16, 2009.

Supplemental material for this article can be found on *http://ajp.amjpathol.org*.

Current address of L.Z.: Laboratory of Petros Papagerakis, School of Dentistry, University of Michigan, Ann Arbor, Michigan.

Address reprint requests to Misako Nakashima, Department of Oral Disease Research, National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, 36-3 Gengo, Morioka, Obu, Aichi 474-8522, Japan. E-mail: misako@nils.go.jp.

MMP-9 to enhance angiogenesis.[12] MMP-2 increases between 7 and 14 days after spinal cord injury, and MMP-2 deficiency results in an extensive astrocytic scar, suggesting MMP-2 promotes functional recovery after injury.[13] Increased expression of MMP-2 and MMP-9 is also associated with the healing mechanism of aneurysm.[14] Gelatinolytic activities corresponding to pro-MMP-2 and the active form of MMP-2 are detected at the wound site 3 and 7 days after glaucoma filtration surgery, suggesting its important roles in the degradation of the ECM in the wound-healing process.[15]

Dental pulp is encased in dentin, which plays a role as a barrier against bacterial, chemical, and physical stimuli. When the barrier is disrupted by traumatic injury or caries, the dentin-pulp complex has a potential to repair and regenerate. Angiogenesis is essential for this pulp wound-healing process, because blood vessels play an important role in nutrition and oxygen supply, as a conduit for transport of metabolic waste, pulp homeostasis and metabolism, and stem/progenitor cell migration.[16] During pulp wound-healing process, dental pulp stem/progenitor cells migrate to the injured site from perivascular region in the pulp tissue deeper from the injured site.[16] They proliferate and differentiate into endothelial cells for angiogenesis/vasculogenesis or into odontoblasts for reparative dentin formation.[17] The angiogenic signals, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor, and transforming growth factor-β, released from injured dental pulp cells, endothelial cells, and ECM by injury contributes to the migration of stem/progenitor cells.[18–20] EphB/ephrin-B also plays a role in restricting dental pulp stem cells attachment and migration to maintain dental pulp stem cells within their stem cell niche under steady-state conditions.[21] Precise mechanism for migrating stem/progenitor cells and angiogenesis/vasculogenesis during pulp wound-healing process, however, still remains unclear.

We hypothesized that certain MMPs expressed during pulp wound healing may support recovery processes. MMP-1, -2, -9, and -14 are expressed in healthy dental pulp.[22] In inflamed pulp, MMP-9 mRNA level is increased in the odontoblasts, fibroblasts, inflammatory infiltrates, and endothelial cells.[23] Expression and function of MMPs during pulp wound healing, however, have not been systematically studied. To address these issues, we established a rat pulp injury model to investigate the expression of MMPs during wound healing. Our results demonstrated that MMP-3 induced the proliferation, migration, and survival (antiapoptosis) of endothelium *in vitro* and accelerated angiogenesis and pulp wound healing *in vivo*.

Materials and Methods

Antibodies

Goat anti-rat CXCR4 from Santa Cruz Biotechnology (Santa Cruz, CA), mouse anti-rat MMP-3 from Daiichi Fine Chemical (Toyama, Japan), normal mouse IgG (AbD Serotec, Oxford, UK), rabbit anti-goat IgG Alexa 488, horseradish peroxidase-labeled goat anti-mouse IgG secondary antibody from Invitrogen (Carlsbad, CA), anti-proliferating cell nuclear antigen (PCNA) antibody from Dako (Carpinteria, CA), and horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody from Zymed Laboratories (San Francisco, CA) were used.

Experimental Pulp Injury Model

Eight-week male Wistar rats, weighing 220–260 g, were purchased (CLEA, Tokyo, Japan). The experimental protocol was approved by the animal committee of the School of Dentistry, Aichi-Gakuin University, and the National Institute for Longevity Sciences of Japan for animal care. The rats were anesthetized with an i.p. injection of sodium pentobarbital (Schering-Plough, Kenilworth, NJ) at a dose of 30 mg/kg, and 2-mm crowns of maxillary incisors were removed with a diamond point burr (Shofu, Tokyo, Japan), then pulp was cut by a no. 1/2 round burr (Shofu). The amputated pulp was thoroughly washed by PBS and covered with spongel (Astellas Pharma, Tokyo, Japan), and the cavities were filled with light-cured composite resin (UniFil Flow GC, Tokyo, Japan) following treatment with a bonding agent (Clearfil Mega Bond; Kuraray, Kurashiki, Japan). One, 12, 24, 72 hours, and 7 days after injury, rats were perfused with 4% paraformaldehyde (Nakarai tesque, Kyoto, Japan) in PBS (pH 7.4), and the incisors with surrounding tissue were extracted with the animals under anesthesia as described previously. The tissues were fixed in 4% paraformaldehyde at 4°C for overnight and decalcified with 10% formic acid for 2 weeks. The tissues were dehydrated in ascending ethanol series and embedded in paraffin wax (Sigma, St. Louis, MO), and 5-μm-thick sections were cut and mounted on aminopropyl triethoxysilane-coated slides (Matsunami, Tokyo, Japan). The slides were stored at 4°C until used for *in situ* hybridization and H&E histological examination. The samples for immunohistochemistry were embedded in optimal cutting temperature compound (Sakura, Tokyo, Japan), and 12-μm-thick frozen sections were cut and mounted on aminopropyl triethoxysilane-coated slides (Matsunami).

Real-Time RT-PCR Analysis

Dental pulp tissues were isolated 0, 12, 24, 48, and 72 hours after injury. The normal pulp tissue from maxillary incisors was used as a control. Total RNA was isolated by TRIzol (Invitrogen) from the freshly excised pulp tissues, and 2 μg of RNA was reverse transcribed with ReverTra Ace-α (Toyobo, Tokyo, Japan), following the manufacturer's recommendations. The resulting cDNA was then amplified by real-time RT-PCR with Light Cycler-FastStart DNA master SYBR Green I (Roche Diagnostics, Mannheim, Germany). Real-time RT-PCR amplifications were performed at 95°C for 10 seconds, 62°C for 15 seconds, and 72°C for 8 seconds using the primers for *β-actin*, *MMP-2*, *MMP-3*, *MMP-9*, *MMP-10*, *MMP-13*, *MMP-14*, *VEGF*, and *CXCR4* (Table 1) labeled with Light Cycler-Fast Start DNA