ニッケルアレルギーの自然免疫を背景とした発症機序

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Lecture

解　説

ニッケルアレルギーの自然免疫を背景とした発症機序*

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はじめに

Niはもっとも頻度の高い接触過敏症のアレルゲンといわれており，Niを含む金属はたくさんある．私たちは種々の経路(生活用品，装飾品，コイン，医・歯科材料など)でNiなどの金属に感作されて金属アレルギーを発症し，また，金属アレルギーは掌跖膿疱症，扁平苔癬，痒疹，紅皮症，扁平上皮癌，さらに自己免疫疾患などとの関連性も示唆されている．金属アレルギーは通常のハプテンの場合と同様にT細胞主役の疾患と考えられているが，その発症機序は明確ではない．グラム陰性菌細胞壁成分のlipopolysaccharide(LPS)は，エンドトキシンとも呼ばれて，私達の生活環境に多少なりとも常に存在する．LPSは自然免疫系を刺激する代表的な物質の一つである．私たちは，このLPSとNiを一緒にマウスに投与すると容易にNiアレルギーが成立することを見出した[1)]．興味深いことに，LPS+Niは獲得免疫を担うT細胞を欠損するマウスにもNiアレルギーを成立させる．最近の実験で，Niアレルギーの感作のステップでは比較的高いNiイオン濃度を要するが，いったん感作が成立し，LPSが存在すると，きわめて低濃度(1×10^{-12}M)のNiイオンでアレルギーが誘導されることがわかった．詳しい解析はこれからであるが，本稿ではNiアレルギーと自然免疫系との関連性について，私たちの実験結果[1)~5)]をもとに考察する．

Niアレルギーの発症機序に関する従来の考え

アレルギー反応は，ある物質に対するsensitization(感作またはpriming)と，その物質により実際に炎症が惹起されるelicitationの2つのステップに区別される．接触過敏症などのアレルギーを起こす種々の低分子量物質をハプテンと呼び，古くから研究で使用されているtrinitrophenolやoxazolonなどのハプテンはその担体になる生体分子と共有結合で結合する．直接ハプテンにはならないが，生体内で変化してハプテン化する物質もある(プロハプテン)．ハプテンが接触アレルギーを誘導する過程は，以下のように考えられている[6)]．担体としては蛋白質が想定されており，皮膚や粘膜とハプテンとの接触で生じたハ

* Innate immunity-associated mechanism underlying nickel-allergy.

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プテン—蛋白複合体は，皮膚ランゲルハンス細胞(LC)やマクロファージなどの抗原提示細胞(APC)に取り込まれて分解され(抗原処理)，生じるペプチドはMHC class IまたはII分子と結合してAPCの細胞表面に出る．このAPCはリンパ節に移動し，多くの未熟なT細胞(naïve T)の中でMHC-ペプチド複合体と構造が適合するものと結合する(抗原提示)．こうして選択されたnaïve Tは活性化され，effecter T細胞とmemory T細胞へ分化・増殖する．これが感作である．感作された動物や私たちが再び同じハプテンに接触すると，そこに分布するLCやマクロファージなどのAPCはsensitizationステップと同様に抗原ペプチドを細胞表面に発現する．Sensitizationが成立している時点ではこの抗原ペプチドと適合するT細胞($CD4^+$および$CD8^+$)はたくさん存在するため，これらの多くが短時間で活性化され，種々のサイトカインやケモカインを放出し結果として種々の細胞が活性化され，$CD8^+$ T細胞による細胞傷害も起こり，アレルギー(炎症)反応が誘導される．これがelicitationステップである．

金属アレルギーも，上記ハプテンによる接触過敏症と同様に，T細胞が関与する疾患と考えられている[7]．しかし，金属イオンは，蛋白と非可逆的な共有結合(covalent bond)で複合体を形成する上記のハプテンと異なり，配位結合(co-ordination bond)で蛋白と結合する．金属イオンの周りに蛋白の極性基(アミノ基，イミダゾール，OH基，SH基など)が立体的に配位する結合であるが可逆的である．したがって，金属イオン—蛋白複合体がその抗原構造(エピトープ構造)を保持したまま抗原処理されて抗原性ペプチドが提示されるとは考えにくい．事実，Niアレルギー患者から分離したT細胞クローンを用いた実験では，抗原処理が不要なクローン，MHC結合ペプチドに特異性を要しないクローン(特定のペプチドに依存しないクローン)，T細胞特異性の低いクローン，金属とある種の蛋白との結合によって構造が変化した蛋白が抗原処理されて出現する抗原性ペプチド(潜在的抗原決定基cryptic epitope)と反応するクローン[8]など，さまざまなクローンが関与する可能性が示唆されている[9]．さらに，ある種の蛋白はNiの単なる“運び屋”として働いて，抗原提示の場でNiを遊離して，T細胞の活性化を効率的にするというシナリオも想定されている[9]．

このように，金属アレルギーでのT細胞の活性化やNi認識の分子機構は多様複雑であるが，いずれにしても，現在，金属アレルギーはT細胞が関与する病気であると認識されている．しかし，金属アレルギー研究のほとんどは，そもそも，すでに感作されたヒトT細胞を用いた*in vitro*を主体とする実験であり，金属アレルギーの成立過程(sensitizationステップ)そのものに関する研究はきわめて少ない．その主な理由は，実験動物に金属アレルギーを起こすことは難しく，金属アレルギーの発症を研究する適切な動物モデルがなかったからと思われる．つまり，金属アレルギーにT細胞が必須であるという基本的な考えに対する実験的証明は，乏しいように思われる．

LPSによるNiアレルギーの促進

モルモットやマウスにNiアレルギーを起こす場合，Ni($NiCl_2$や$NiSO_4$)のみによる感作はなかなか成功しない．アジュバントとしてcomplete Freund's adjuvant(CFA)を使っても，20～200mMの高濃度を必要とする[10][11]．この濃度ではNiイオンそのものの毒性が強く，さらに，CFA自体の傷害性も加わって，注射部位は壊死やケロイド状態になる．また，0.6MH_2O_2をアジュバントとし20mM$NiCl_2$を使用するマウスモデルもあるが[12]，この場合も注射部位はケロイド状態になる．つまり，実験動物を感作するには，ヒトでの実際の病気の場合とはほど遠い激しい条件が必要とされる．これらのモデルは，ヒトでのNiアレルギーの成立過程を研究する適切なモデルとはいえないのではないか？と私たちは考えた．なお，H_2O_2をアジュバントとして用いる実験では，Niの少ない環境で2世代飼育したマウスでは1 mM $NiCl_2$でも感作が成立することが最近報告されている[13]．

LPSはグラム陰性菌細胞壁成分であり，エンドトキシンとして研究されている．論文としての発表は遅れたが，LPSはマウスを卵白アルブミンで感作する際の強いアジュバントになることを

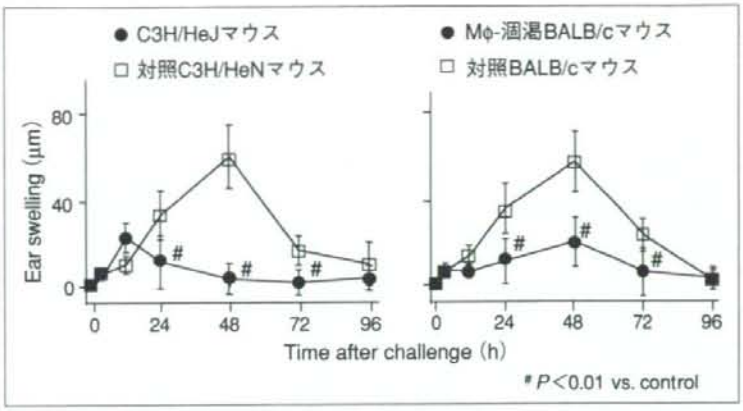

図1　Ni(＋LPS)-allergyとTLR4とマクロファージ(Mφ)との関係
LPS(1 μg/ml)とNiCl₂(1 mM)の等量混合液0.25mlを腹腔中に注射し，10日後，NiCl₂(5 mM)20μlを耳に皮内注射(challenge)した．耳での腫脹をアレルギー反応として評価した．測定値は平均値±SD．　(文献[1]より引用改変)

私たちは観察していた[14]．このLPSとNiCl₂との併用により，sensitizationステップで，マウス組織に炎症や，ケロイド，壊死などを起こすことなく，Niアレルギーを誘導することに成功した[1]．LPS(1 μg/ml)とNiCl₂(1 mM)の等量混合液0.25mlを腹腔注射することにより，マウス(BALB/c, C57BL/6, C3H/HeN)は確実にNiに対して感作される．私たちは，便宜上このモデルをNi(＋LPS)-allergyと呼称している．LPSはNiのみならず，Cr, Co, Pd, Agなどに対するsensitizationも促進する．

LPSはsensitizationステップのみならず，elicitationステップでもNiアレルギーを促進する[1]．最近の実験で[2]，sensitizationステップでの最少NiCl₂濃度は，LPSなしで1×10^{-2}Mであるが，LPSプラス(～50μg/ml)では1×10^{-5}～10^{-3}Mであった．また，elicitationステップでの最少有効NiCl₂濃度は，LPSなしで1×10^{-6}Mであり，LPSプラス(1 μg/ml)では1×10^{-12}Mという超低濃度であった．これらの結果は以下を示唆する．Niアレルギーでのsensitizationステップでは高いNi濃度を要するが，いったん感作が成立すると，きわめて低濃度でアレルギーを誘導する．しかし，これらのステップでLPSがNiアレルギーを促進する分子機序は不明である．

Ni(＋LPS)-allergyに関与する自然免疫関連分子

菌体成分は細胞表面や細胞内の種々のToll-like receptors(TLRs)との結合を介して免疫反応を誘導し，LPSは細胞表面のTLR4と結合して細胞を刺激する．したがって，TLRsは自然免疫におけるきわめて重要な分子である[15][16]．C3H/HeJマウスはTLR4が変異したマウスで，LPS-non(or low)-responderとして知られている．このマウスでは，上述のNi(＋LPS)-allergyはきわめて弱い[1](図1)．TLRsシグナル経路はIL-1受容体シグナル経路とよく似ている[16]．事実，IL-1-knockout(KO)マウスでもNi(＋LPS)-allergyはわずかであった．しかし，TNF-α-KOマウスでは，野生型マウスと同様にNi(＋LPS)-allergyが誘導された[1]．

LPS以外の菌体成分の効果

私たちの体内に存在する免疫担当細胞は自然免疫を担う種々の分子により菌体や菌体成分を認識して，免疫応答を惹起する．TLRsはこれらを認識する重要な分子群であり，TLRsによるシグナル伝達機構は，互いによく似ている[16]．このことは，他の菌体成分や関連物質もLPSと同様にNiアレルギーを促進することを予想させる．私たちは現在，sensitizationステップとelicitationステップの各段階でのこれらの物質の効果を調べ

表1 Sensitizationステップでニッケルアレルギー促進効果を示す細菌および関連物質の最小有効濃度

被試験物質		最少有効濃度 (μg/ml)
LPS		
Escherichia coli	TLR4-ligand	0.01
Prevotella intermedia	TLR4-ligand＋TLR2-ligand?	0.01
Pam3Cys-SKKKK	Synthetic TLR2-ligand	10
Mannan	TLR4-ligand and/or TLR2-ligand	10
Poly I:C	TLR3-ligand	1,000
Propionibacterium acnes	Heat-killed gram(＋) bacterium	>1,000
MDP	NOD-2-ligand	1,000
Bisphosphonates		
Alendronate	Chemical substance (IL-1-producing)	200
Zoldronate	Chemical substance (IL-1-producing)	100
Concanavalin A	T cell mitogen (IL-1-producing)	1,000

種々の濃度の被検物質と$NiCl_2$(1 mM)の等量混合液0.25mlをBALB/cマウス腹腔に注射し，10日後，$NiCl_2$(5 mM)20μlを耳に皮内注射(challenge)した．Challenge後の耳での腫脹反応をもとに被検物質の最少有効濃度を求めた．

ている．これまで，表1に示した種々の炎症性物質がsensitizationステップでニッケルアレルギーを促進することがわかった[3]．これらの物質の多くはelicitationステップにおいてもニッケルアレルギーを促進した[4]．Mannanは真菌の成分であるが，かなり強いアジュバント効果を示した．したがって，グラム陰性菌のみならず，真菌なども金属アレルギーの発症を促進する要因となる可能性がある．

Ni(＋LPS)-allergyに関与する細胞

ヌードマウスはT細胞を欠損し，SCIDマウスはT細胞とB細胞を欠損する．前述のように，金属アレルギーはT細胞に依存する病気と考えられている．しかし，驚いたことに，これらのマウスでも野生型マウスと同様にNi(＋LPS)-allergyが誘導された(図2)．この結果は，Ni(＋LPS)-allergyの成立には，獲得免疫を担うT細胞よりも，自然免疫系を担う細胞がきわめて重要であることを示している．通常のニッケルアレルギーには自然免疫系を担う細胞とT細胞の両者が関与するが，T細胞がなくてもニッケルアレルギーは起こりうるということであろうか？

驚いたことに，最近O'Learyら[17]は，代表的なハプテンである2,4-dinitrofluorobenzene(DNFB)とoxazolone(Oxa)による接触アレルギーも，T細胞やB細胞を欠損するマウスに誘導され，これらのハプテンによるアレルギーには，NK細胞が重要であると報告している．また，かなり特殊な実験モデルであるが，trinitrophenol(TNP)特異的IgEトランスジェニックマウスにTNP結合卵白アルブミン(TNP-OVA)を耳介皮内注射して生じるIgE依存性の慢性アレルギーにおいてもT細胞は必須ではなく，好塩基球が主役であることが示されている[18][19]．

マクロファージは主要な抗原提示細胞である．Clodronateという薬物を封入したliposome(脂質微粒子)を貪食したマクロファージは，すみやかに死滅するため，clodronate-liposomeはマクロファージやmonocytesの涸渇に用いられている[20]．この方法でマクロファージを涸渇したマウス(clodronate-liposomeを静脈注射24時間後にLPS＋Niで感作)においては，Ni(＋LPS)-allergyの発症はわずかであった[1](図1)．

上述のO'Learyらの論文[17]に触発されて，私たちもNi(＋LPS)-allergyにおけるNK細胞の関与を検討した．抗asialo-GM1抗体はNK細胞を涸渇することが知られている．この方法でNK細胞を涸渇した群と涸渇しない群をLPS＋Niで感作し，アレルギー反応をBALB/cとヌードBALB/cマウスで比較した(図3)．BALB/cではNK細胞の涸渇によりアレルギー反応は半減し，ヌードBALB/cマウスではNKがなくなるとアレルギー反応はまったく起こらなかった．この結果は，野生型マウスでのNi(＋LPS)-allergyにおいては，NK細胞が

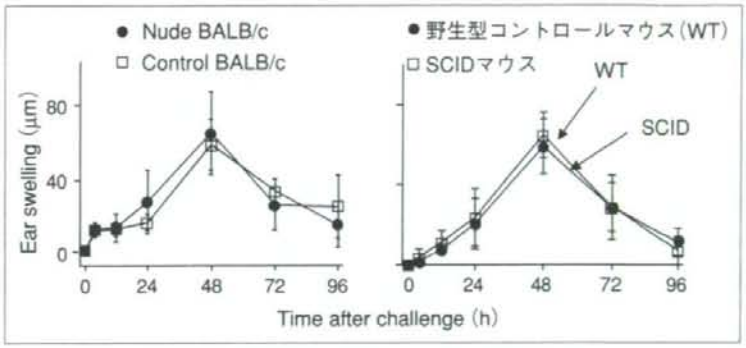

図2　Ni(＋LPS)-allergyとT細胞との関係
それぞれのマウスについて図1と同様に実験した．(文献[1]より引用改変)

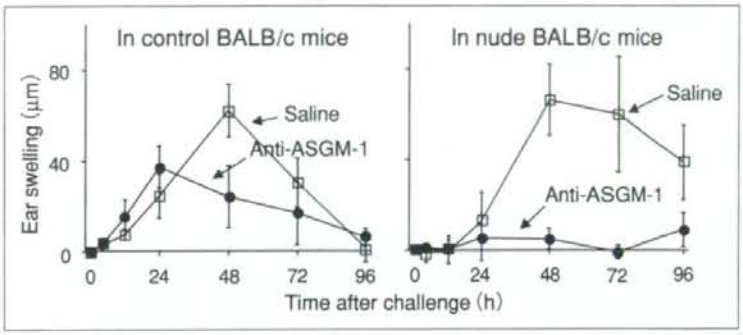

図3　Ni(＋LPS)-allergyでのNK細胞枯渇の効果
マウスに抗asialo GM1 polyclonal抗体を静脈注射し(0.2mg/mouse)，24時間後，図1と同様に感作注射を行い，その3日後，再度抗asialo GM1 polyclonal抗体を1回目と同様に注射した．感作注射の10日後，図1の実験と同様にchallengeを行い，腫脹を測定した．

部分的に関与し，T細胞欠損マウスではNKが必須であることを示唆している．しかし，抗asialo-GM1抗体はmonocytesやマクロファージなど他の細胞にも弱いながら反応性を示すとのことであり，また，好塩基球も反応するということから，結論には詳細な解析が必要である．

ヒスタミンの関与

LPSはきわめて低い用量で，マウスの種々の組織にヒスタミン合成酵素のhistidine decarboxylase (HDC)を誘導する[21][22]．IL-1も種々の組織にHDCを誘導し，炎症性サイトカインの中では最強のHDC誘導作用をもつ[23]．LPSとIL-1はマスト細胞を欠損するマウスにおいても種々の組織にHDCを誘導する[24]．一方，多くの研究で，ヒスタミンはTh1/Th2反応の調節因子であることが示されている[25]~[27]．ヒスタミンとNi(＋LPS)-allergyとの関連性について，私たちは以下の結果を得ている．①マスト細胞欠損マウスでも同腹対照マウスと同等またはそれ以上にアレルギー反応が起こる[1]．②ElicitationステップにおいてHDC活性は炎症の進行とパラレルに亢進する[1]．③HDC-KOマウスでのアレルギー反応は野生型マウスに比べ弱い[1]．そこで，野生型とHDC-KOマウスの両方を感作して，これらのマウスの脾臓細胞を感作していない野生型とHDC-KOマウスに移入して，アレルギーが起こるかどうかを調べると，野生型マウスに移入した場合は，どちらのマウスの細胞も同じ程度にアレルギーを起こすが，HDC-KOマウスへの移入では，どちらの細胞もアレルギーを起こさない[5]．④ヒスタミン受容体H1-KOマウスでも，アレルギー反応はほとんど

起こらない．上の実験と同様に，野生型とH1-KOマウスの両方を感作して，これらのマウスの脾臓細胞を感作していない野生型とH1-KOマウスに移入してアレルギーが起こるかどうかを調べると，野生型マウスに移入した場合は，どちらのマウスの細胞も同じ程度にアレルギーを起こしたが，H1-KOマウスへの移入では，どちらの細胞もアレルギーを起こさない[5]．これらの結果は，①ヒスタミンは，H1受容体を介して，sensitizationのステップではなく，elicitationのステップに関与し，②そのヒスタミンはマスト細胞由来ではなく，HDC誘導により産生されるヒスタミンであることを示唆している．抗ヒスタミン薬の臨床応用を支持する結果であるが，その有効性は著明ではないと認識されているようである．しかし，劇的な効果をもたらしたという症例を経験しており（未発表），今後の積極的な評価を期待したいところである．

Ni(＋LPS)-allergyの交差反応

Ni＋LPSで感作したマウスでは，Ni, Pd, Cr, Co, Cu, Ag, いずれも数μMの濃度でアレルギーを誘導し，Pd, Cr, AgなどはNiよりもむしろ強いアレルギー反応を誘導した[2]．Cuはそれ自体が他の金属にはみられない炎症反応を誘導し，この炎症が起こらない低濃度でNiと交差反応を示した．LPSは交差反応でのこれらの金属の最少有効濃度も著しく低下させた．交差反応でのPd, Cr, Coでの最少有効濃度はLPSなしで1×10^{-6}Mのレベルであるが，LPSプラス（1 μg/ml）では1×10^{-14}～10^{-12}Mのレベルまで低下した．これらの結果は，金属イオン—蛋白複合体の立体構造は，金属が違っても共通性をもち，共通な抗原となりうる可能性を示唆する．これまで予想されていない興味深い知見である．しかし，上で述べたように，elicitationのステップでは，きわめて低濃度の金属イオンがアレルギー反応を誘導することを考えると，観察した交差反応は実験に使用した金属塩試薬に微量混入する他の金属による可能性を排除できない．したがって，上記のNiアレルギーの広い交差反応を結論するには，超高純度の試薬を用いた注意深い検討が必要であると考えている．

おわりに

上に述べてきたように，接触アレルギーは，必ずしも，従来考えられてきたT細胞主役の機序だけで起こるわけではなさそうであり，これまでの概念が揺らぎつつあるようである．金属イオンの場合は，担体蛋白と可逆的な配位結合で結合し，通常のハプテンに比べて，アレルギーの発症機序は，さらに複雑らしい．私たちの体は，従来考えられてきた機序以外にも，多くの手段を用いて，免疫反応を行っているらしい．体内も含めて細菌は生活環境の至る所に存在し，免疫系は細菌と共存の環境で進化してきた．獲得免疫以前に自然免疫があり，進化した現在でも両方のシステムが重要な役わりを果たしていると思われる．金属という“文明社会が生み出した異物”に対して，私たちの体に今なお残る太古の免疫システムも反応しているようである．そして，従来の概念にとらわれない研究が必要と思われる．

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Dual regulation of interleukin-8 production in human oral epithelial cells upon stimulation with gingipains from *Porphyromonas gingivalis*

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Cysteine proteinases from *Porphyromonas gingivalis*, or gingipains, are considered to be key virulence factors of the bacterium in relation to periodontal diseases. Incubation of human oral epithelial cells with lysine-specific gingipain (Kgp) and high-molecular-mass arginine-specific gingipain (HRgpA) resulted in a decrease in the production of interleukin (IL)-8, but not in the production of other pro-inflammatory cytokines. In contrast, arginine-specific gingipain 2 (RgpB) increased IL-8 production. RNA interference assays demonstrated that Kgp- and HRgpA-mediated downregulation and RgpB-mediated upregulation occurred through protease-activated receptor (PAR)-1 and PAR-2 signalling. Although the RgpB-mediated upregulation of IL-8 production occurred through nuclear factor-kappa B (NF-κB), the Kgp- and HRgpA-mediated downregulation was not negated in NF-κB-silenced cells. Both the haemagglutinin and the enzymic domains are required for Kgp and HRgpA to downregulate the production of IL-8 in human oral epithelial cells, and the two domains are thought to co-exist. These results suggest that gingipains preferentially suppress IL-8, resulting in attenuation of the cellular recognition of bacteria, and as a consequence, sustain chronic inflammation.

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INTRODUCTION

Periodontitis is an inflammation of the whole periodontium. The dominant cells in periodontal epithelial tissue are the oral epithelial cells. The barrier function of oral epithelial cells is mainly due to the production of antimicrobial peptides, such as human β-defensins and cathelicidin (Acheson & Luccioli, 2004). Healthy gingival epithelium is characterized by the presence of human β-defensin-2 and a gradient of interleukin (IL)-8 that guides the transmigration of leukocytes (Dixon *et al.*, 2004; Pütsep *et al.*, 2002). Inflamed gingival epithelium is severely infiltrated with leukocytes around the periodontal pocket (gingival crevice), accompanied by elevated expression of IL-8 (Liu *et al.*, 2001). Chemokines such as IL-8 form the first line of host defence by increasing phagocytosis, bacterial killing, the release of lysosomal enzymes and superoxide anion generation (Weiss, 1989), indicating that this mechanism is of great importance for innate immunity. *Porphyromonas gingivalis* has been implicated not only in severe chronic periodontitis, but also in aggressive periodontitis (Holt & Bramanti, 1991). *P. gingivalis* possesses a number of putative virulence factors, such as LPS, fimbriae and proteinases (Chen *et al.*, 1992). We have studied the virulence activities of two types of trypsin-like cysteine proteinase (Potempa *et al.*, 1995) that cleave specifically at Arg–X (50 and 95 kDa) (Wingrove *et al.*, 1992) and Lys–X (105 kDa) bonds and are referred to as arginine-specific gingipain (Rgp) and lysine-specific gingipain (Kgp), respectively (Pike *et al.*, 1994). The

Abbreviations: FPR-cmk, Phe-Pro-Arg-chloromethyl ketone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRgpA, high-molecular-mass arginine-specific gingipain; IFN-γ, gamma interferon; IL, interleukin; Kgp, lysine-specific gingipain; NF-κB, nuclear factor-kappa B; PAR, protease-activated receptor; Rgp, arginine-specific gingipain; PAR-1AP, PAR-1 agonist peptide; PAR-2AP, PAR-2 agonist peptide; PAR-3AP, PAR-3 agonist peptide; siRNA, short interfering RNA; z-FKck, benzyl-oxycarbonyl-Phe-Lys-chloromethyl; TNF-α, tumour necrosis factor alpha.

95 kDa high-molecular-mass arginine-specific gingipain (HRgpA) and Kgp are a complex of the catalytic domain and the haemagglutinin/adhesin domain, and differ from the 50 kDa Rgp (RgpB), which lacks the latter domain. We have shown that gingipains cleave CD14 on human monocytes (Sugawara *et al.*, 2000) and gingival fibroblasts (Tada *et al.*, 2002), and ICAM-1 on human oral epithelial cells (Tada *et al.*, 2003), inhibiting the LPS-elicited defensive response of these cells against this pathogen, and interaction between epithelial cells and leukocytes, respectively, which facilitates *P. gingivalis* in evading the innate immunity. Thus, gingipains are important virulence factors of *P. gingivalis*.

Members of the protease-activated receptor (PAR) family are G protein-coupled receptors (Coughlin, 2000; Déry *et al.*, 1998; O'Brien *et al.*, 2001). There are four members of this family. As PARs are expressed in a wide variety of cell types, it was recently suggested that they may play important roles in pathophysiological processes such as growth, development, inflammation, tissue repair and pain (Coughlin, 2000; Déry *et al.*, 1998; O'Brien *et al.*, 2001). In fact, we demonstrated that neutrophil serine proteinase 3 activates human oral epithelial cells and human gingival fibroblasts via the PAR-2 pathway (Uehara *et al.*, 2003, 2002b). It has been reported that RgpB cleaves and activates PAR-2 on human neutrophils (Lourbakos *et al.*, 1998), induces IL-6 secretion by activating PAR-1 and PAR-2 on human oral epithelial KB cells (Lourbakos *et al.*, 2001a), and causes platelet aggregation via PARs (Lourbakos *et al.*, 2001b). Furthermore, RgpB induces neuropeptide release from dental pulp cells via PAR-2 signalling (Tancharoen *et al.*, 2005). Recently, we revealed that Rgps (HRgpA and RgpB) stimulated the production of hepatocyte growth factor through PAR-1 and PAR-2 in human gingival fibroblasts (Uehara *et al.*, 2005), which may be associated with both inflammatory and reparative processes of periodontal disease. PARs are important molecules that mediate gingipain stimuli to cells. In this study, we analysed the effects of gingipains on the production of cytokines, particularly IL-8, by oral epithelial cells and investigated the possible involvement of PARs in gingipain effects.

METHODS

Reagents. Human natural gamma interferon (IFN-γ) was provided by Hayashibara Biochemical Laboratories. Human recombinant (r)IL-1α and recombinant tumour necrosis factor alpha (TNF-α) were supplied by Dainippon Pharmaceutical. Phe-Pro-Arg-chloromethyl ketone (FPR-cmk) and benzyloxycarbonyl-Phe-Lys-chloromethyl (z-FKck) were obtained from Bachem Bioscience. PAR-1 agonist peptide (PAR-1AP; SFLLRN), PAR-2 agonist peptide (PAR-2AP; SLIGKV) and PAR-3 agonist peptide (PAR-3AP; TFRGAP) were synthesized by Takara. All other reagents were obtained from Sigma-Aldrich, unless otherwise indicated.

Purification and activation of gingipains. Three forms of gingipain – 95 kDa HRgpA, 50 kDa RgpB and 105 kDa Kgp – were purified from *P. gingivalis* HG66 culture supernatant, as described previously (Pike *et al.*, 1994; Potempa *et al.*, 1998). The purity of each enzyme was checked by SDS-PAGE. In a 10% Tricine gel, RgpB migrated as a single band with a mobility equivalent to a molecular mass of 48 kDa and homogeneity greater than 95% as determined using laser densitometric scanning of the gel. HRgpA resolved into four major bands and one minor band on SDS-PAGE (Pike *et al.*, 1994). The identity of each protein band was confirmed by N-terminal sequence analysis as being derived from the HRgpA polyprotein. The amount of active enzyme in each purified gingipain was determined by active-site titration using FPR-cmk and z-FKck for the Rgps and Kgp, respectively (Potempa *et al.*, 1997). The concentration of fully activated gingipain with cysteine was calculated from the amount of inhibitor needed for complete inactivation of the proteinases. Therefore, the concentrations of gingipains indicated in this paper are represented as those of active gingipains. The gingipains were activated by diluting to 10 μM in 0.2 M HEPES (pH 8), 5 mM $CaCl_2$ and 10 mM cysteine, and incubating at 37 °C for 10 min. The activated gingipains were then diluted with medium or buffer. To block their enzymic activity, the activated gingipains were incubated with the specific inhibitors FPR-cmk and z-FKck for 10 min at room temperature prior to use.

Construction of a strain producing Kgp proteinase without the adhesin domains in a Rgp/Kgp/adhesin-null mutant. The 1.6 kb *Eco*RV–*Sma*I DNA fragment (*cepA* DNA block) of pCS22 (gift from Dr Christine Seers, Cooperative Research Centre for Oral Health Science, School of Dental Science, University of Melbourne, Australia) was inserted into the *Sma*I site of pKD703 (Shoji *et al.*, 2004) with and without the 6.7 kb *Xho*I–*Not*I DNA fragment (*kgp'–'rgpB* chimeric gene DNA, blunt-ended) of pKD855 (Sato *et al.*, 2005) to yield plasmids pKD856 (*fimA*::[*kgp'–'rgpB cepA*]) and pKD857 (*fimA*::*cepA*), respectively. *Sca*I-linearized pKD856 or pKD857 was introduced into the Rgp/Kgp/adhesin-null mutant KDP153 (Naito *et al.*, 2006), resulting in the transformants KDP154 and KDP160, respectively. KDP160 produces Kgp proteinase without the adhesin domains from the *kgp'–'rgpB* chimeric gene.

Collection of supernatants of wild-type *P. gingivalis* and mutant *P. gingivalis* strains and preparation of the adhesin domains rHgp44 and rHbR. *P. gingivalis* 33277 was grown anaerobically to stationary phase in enriched brain heart infusion broth with menadione and haemin without antibiotics. Mutant strains *P. gingivalis* KDP133 (*rgpA rgpB*), *P. gingivalis* KDP136 (*rgpA rgpB kgp*) (Shi *et al.*, 1999), *P. gingivalis* KDP137 (*rgpA kgp hagA*) (Shi *et al.*, 1999), *P. gingivalis* KDP153 (*rgpA rgpB kgp hagA*), KDP160 (*rgpA rgpB kgp hagA fimA*::[kgp'–'rgpB]) and KDP161 (*rgpA rgpB kgp hagA fimA*) (Table 1) were grown anaerobically to stationary phase in enriched brain heart infusion broth with menadione, haemin and erythromycin (10 μg ml^{-1}). After 2 days of culture, supernatants were obtained by centrifugation at 10000 *g* for 20 min at 4 °C and

Table 1. Phenotype of protein fractions prepared from culture supernatants of wild-type and various mutant *P. gingivalis* strains

Strain	*rgpA*	*rgpB*	*kgp*	*hagA*
33277	+	+	+	+
KDP133	–	–	+	+
KDP137	–	+	–	–
KDP136	–	–	–	+
KDP153	–	–	–	–
KDP160	–	–	+	–
KDP161	–	–	–	–

then precipitated with 75% saturated ammonium sulphate at 4 °C for 1 h. The precipitate was collected by centrifugation at 10 000 *g* for 20 min at 4 °C and then dialysed three times against 0.05% Brij35 (Sigma-Aldrich) in 10 nM sodium phosphate buffer (pH 7) at 4 °C. The procedure used to prepare supernatants of *P. gingivalis* was based on the purification technique for gingipain as described in detail previously (Kadowaki *et al.*, 1994). Purification of the rHgp44 and rHbR (Hgp15) adhesin proteins was conducted as described previously (Naito *et al.*, 2006; Nakayama *et al.*, 1998).

Cells and cell culture. Human oral epithelial cell line HSC-2 (Momose *et al.*, 1989), established from a squamous cell carcinoma, was obtained from the Cancer Cell Repository, Institute of Development, Ageing and Cancer, Tohoku University, Japan. HSC-2 cells were grown in RPMI 1640 (Nissui Seiyaku) with 10% heat-inactivated fetal calf serum (Life Technologies) with a change of medium every 3 days. To avoid the possibility of trypsinization affecting the amounts of PAR and other surface markers, we used cell dissociation solution (Sigma-Aldrich), which contains no protein and allows the dislodging of cells without the use of enzymes; thus, cellular proteins are preserved without enzymic modification or the adsorption of foreign proteins.

ELISA of cytokines. Cells (1×10^4 in 200 µl) were incubated with or without stimulant in RPMI 1640 with 10% fetal calf serum for 24 h in 96-well, flat-bottomed plates (Falcon). Culture supernatants were collected and levels of IL-8 were determined using an ELISA kit (BD Pharmingen). The concentrations of cytokines in the supernatants were determined using the LS-PLATE 2004 data analysis program (Wako Pure Chemical Industries).

RT-PCR assay. Total cellular RNA was obtained using Isogen (Nippon Gene), and was reverse transcribed using random hexamer primers and avian myeloblastosis virus transcriptase XL. The primers used for PCR were as follows: IL-8, 5′-GATTGAGAGTGGACC-ACACT-3′ and 5′-TCTCCCGTGCAATATCTAGG-3′; and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-TGAAG-GTCGGAGTCAACGGATTTGGT-3′ and 5′-TGAAGGTCGGAGT-CAACGGATTTGGT-3′, generating fragments of 422 and 286 bp, respectively. Cycling conditions were 25 cycles of 94 °C for 1 min, 63 °C for 1 min and 72 °C for 1 min. Amplified samples were visualized on 2% agarose gels stained with ethidium bromide and photographed under UV light.

RNA interference. Transfection for targeting endogenous PAR-1, PAR-2 and nuclear factor-kappa B (NF-κB) subunit p65 was carried out using Lipofectamine 2000 (Invitrogen) and short interfering RNA (siRNA) (final concentration 200 nM), according to the manufacturer's instructions. siRNA of PAR-1, PAR-2 and NF-κB p65, and anti-NF-κB p65 antibody (mouse IgG2a), were purchased from Santa Cruz Biotechnology.

NF-κB activity. Activated NF-κB was measured with an NF-κB assay kit specific for the p65 subunit according to the manufacturer's instructions (Active Motif). Briefly, samples of whole-cell extracts (1–10 µg protein per well) were added to 96-well plates coated with an oligonucleotide containing the NF-κB consensus site (5′-GGGACTTTCC-3′) and incubated for 1 h at room temperature with mild agitation. After three washes, NF-κB p65 antibody was added for 1 h without agitation, followed by horseradish peroxidase-conjugated anti-mouse IgG1. Colorimetric reactions were developed and stopped and the absorbance measured at 450 nm. The specificity of binding was also examined using an oligonucleotide containing a wild-type or mutated NF-κB consensus binding site.

Data analysis. Statistical significances were determined using ANOVA with the Bonferrori or Dunnett method.

RESULTS AND DISCUSSION

The oral epithelium is directly exposed to periodontal bacteria, and their products may play an important role in host defence mechanisms against pathogens. To investigate the effects of gingipains (HRgpA, RgpB and Kgp) on the secretion of IL-8 from human oral epithelial cells, HSC-2 cells were incubated with gingipains. We found a dual effect of gingipains on IL-8 secretion of oral epithelial cells: a decrease by complex forms carrying haemagglutinin/adhesin domains (Kgp and HRgpA) and an increase by a form lacking the haemagglutinin domains (RgpB) (Fig. 1). Spontaneous IL-8 secretion was decreased by Kgp and HRgpA at concentrations of 20–200 nM after 24 h incubation, but, in contrast, was increased by RgpB at 200 nM (Fig. 1a). The downregulation by Kgp and HRgpA and the upregulation by RgpB were also significant after a 12 h incubation, reaching a maximum at 24 h and continuing until 48 h (Fig. 1b). RNA was then extracted from oral epithelial cells stimulated with gingipains and RT-PCR was performed to define the level of IL-8 mRNA. IL-8 mRNA was expressed in untreated cells and the expression of IL-8 mRNA was significantly downregulated by Kgp and HRgpA, but not by RgpB (Fig. 1c). It should be noted that Kgp and HRgpA did not cause a decrease in the production of other pro-inflammatory cytokines (IL-1α, IL-6, monocyte chemoattractant protein-1 and TNF-α) (data not shown). In contrast, PAR-1AP and PAR-2AP, but not PAR-3AP, clearly upregulated IL-8 production (Fig. 1d), which was consistent with results of our previous studies (Uehara *et al.*, 2002b, 2004). We also examined whether Kgp and/or HRgpA was capable of inhibiting enhanced IL-8 production induced by pro-inflammatory cytokines in oral epithelial cells. As shown in Fig. 2, Kgp and/or HRgpA clearly inhibited the production of IL-8 induced by IFN-γ, IL-1α or TNF-α in oral epithelial cells almost to baseline level. The findings suggested outstanding downregulatory effects of gingipains on IL-8 secretion. It must be noted here that similar results to those obtained using HSC-2 cells in Figs 1 and 2, and in additional experiments, were also obtained from primary oral epithelial cells and two other oral epithelial cell lines (data not shown).

We then examined whether the Kgp- and HRgpA-mediated downregulation and RgpB-mediated upregulation of IL-8 production in oral epithelial cells were due to the enzymic activities of gingipains. It has been reported that the enzymic activity of Rgp and Kgp are inhibited specifically by FPR-cmk and z-FKck, respectively (Potempa *et al.*, 1997). FPR-cmk, a specific inhibitor of Rgps, almost completely negated the RgpB-induced upregulation and HRgpA-induced downregulation (Fig. 3). z-FKck, a specific inhibitor of Kgp, also clearly inhibited the Kgp-mediated downregulation of IL-8 production (Fig. 3). These results indicated that RgpB-mediated upregulation, Kgp-mediated downregulation and HRgpA-mediated downregulation of IL-8 production were dependent on their enzymic activities.

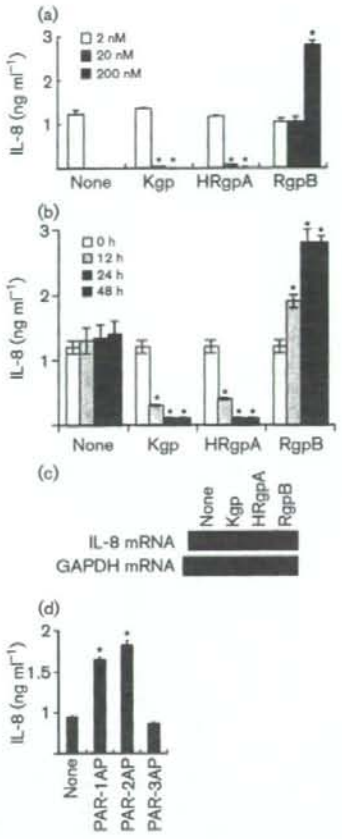

Fig. 1. Dual regulation of IL-8 secretion by gingipains and PAR agonist peptides in human oral epithelial cells in culture. (a, b, d) HSC-2 cells were stimulated with gingipains (Kgp, HRgpA or RgpB) at the concentrations indicated for 24 h (a), with 200 nM gingipains for the periods indicated (b), or with PAR agonist peptides (200 μM) for 24 h (d), in triplicate at 37 °C. Activating solution for gingipains (a, b) was used as a control. IL-8 levels in the culture supernatants were determined by ELISA and expressed as means ± SD. *, $P<0.01$ compared with medium alone. (c) HSC-2 cells were stimulated without or with 200 nM gingipain (Kgp, HRgpA or RgpB) for 6 h, and the expression of IL-8 and GAPDH mRNA was analysed by RT-PCR.

We next examined whether downregulation and/or upregulation of IL-8 production also occurred through PAR family members. As demonstrated previously, human oral epithelial cells constitutively express mRNAs and cell-surface proteins of PAR-1, PAR-2 and PAR-3 but not PAR-4 (Uehara *et al.*, 2002b). To block PAR expression on oral epithelial cells, we used siRNAs for PARs. As shown previously (Uehara *et al.*, 2005), transfection of human oral epithelial cells with PAR-1-, PAR-2- or PAR-3-specific siRNA results in an approximately 80 % decrease in the

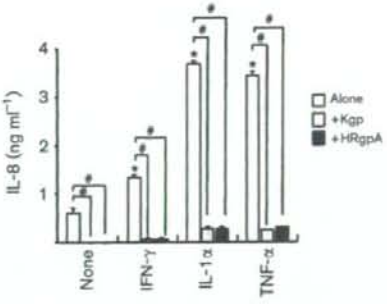

Fig. 2. Downregulation of IL-8 secretion induced by inflammatory cytokines in human oral epithelial cells in culture. HSC-2 cells were incubated with or without IFN-γ (1000 IU ml^{-1}), IL-1α (10 ng ml^{-1}) or TNF-α (10 ng ml^{-1}) for 24 h. After three washes with PBS, cells were incubated for 24 h in the presence or absence of gingipain (200 nM) at 37 °C. IL-8 concentration was determined by ELISA. * and # indicate that values differ significantly compared with the respective controls.

level of PAR-1, PAR-2 or PAR-3 mRNA, but not GAPDH mRNA, in cells cultured for 24–72 h. In both PAR-1- and PAR-2-siRNA transfected cells, Kgp- and HRgpA-mediated downregulation was significantly eliminated and RgpB-mediated upregulation was also suppressed, but the effect of PAR-3-siRNA transfection was not significant (Fig. 4). The decrease in IL-8 production by Kgp and HRgpA in epithelial cells is believed to be the first report that gingipains inhibit cellular function via PARs.

In our previous study (Uehara *et al.*, 2005), the Rgp-induced production of hepatocyte growth factor occurred via NF-κB downstream of PAR signalling. As shown in Fig. 5(a), Kgp, HRgpA and RgpB significantly increased

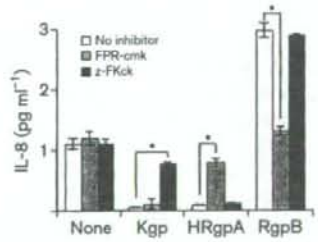

Fig. 3. Effect of specific inhibitors for gingipains, cytochalasin B and cycloheximide, on gingipain-mediated regulation of IL-8 secretion. Gingipains were pre-treated with FPR-cmk (10 μM) or z-FKck (100 μM) for 15 min at 37 °C before use. HSC-2 cells were stimulated with or without gingipains for 24 h. Activating solution for gingipains was used as a control. IL-8 levels in the culture supernatants were determined by ELISA and are expressed as means ± SD. *, $P<0.01$ compared with the respective control.

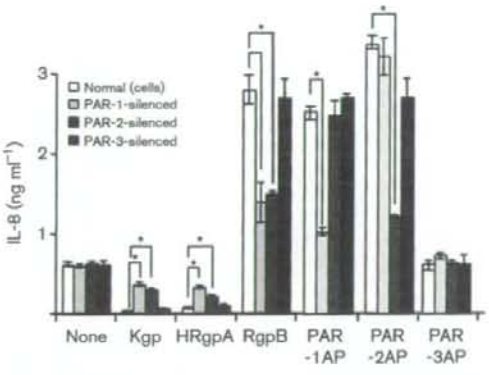

Fig. 4. Involvement of PAR-1 and PAR-2 in gingipain-mediated downregulation of IL-8 production in human oral epithelial cells. HSC-2 cells were transfected with PAR-1-, PAR-2- or PAR-3-specific-siRNA for 24 h, and incubated for an additional 24 h in the presence or absence of gingipains (200 nM) at 37 °C. PAR-1AP, PAR-2AP and PAR-3AP were used as reference stimulants. Activating solution for gingipains was used as a control. IL-8 concentrations were determined by ELISA. *, $P<0.01$ compared with the respective control.

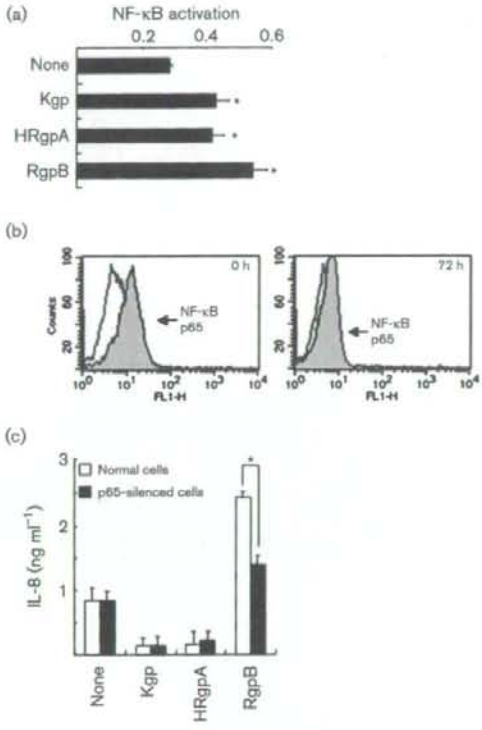

Fig. 5. NF-κB is not involved in gingipain-mediated downregulation of IL-8 production in human oral epithelial cells. (a) HSC-2 cells were incubated for 1 h in the presence or absence of gingipains (200 nM) at 37 °C and active NF-κB was determined by ELISA. Activating solution for gingipains was used as a control. (b) HSC-2 cells were transfected with siRNA for NF-κB p65. After 0 and 72 h, the cells were stained with anti-NF-κB p65 antibody at 4 °C for 30 min, followed by FITC-conjugated goat anti-mouse IgG. (c) HSC-2 cells transfected with p65-specific-siRNA for 24 h were stimulated with gingipains (200 nM). Activating solution for gingipains was used as a control. After 24 h of stimulation, IL-8 concentration in the culture supernatants was determined by ELISA. All samples were assayed in triplicate and the results are expressed as means ± SD. *, Significant difference compared with the respective control.

active NF-κB in human oral epithelial cells. Therefore, we examined the possible involvement of NF-κB in the regulation of IL-8 by gingipains using siRNA targeting p65, which is a component of NF-κB. NF-κB p65 protein levels determined by flow cytometry were reduced by approximately 80 % using specific siRNA in HSC-2 cells up to 72 h (Fig. 5b). Upregulation of IL-8 production by RgpB was significantly inhibited in p65-silenced oral epithelial cells (Fig. 5c). However, downregulation of IL-8 production by Kgp and HRgpA was not inhibited in p65-silenced cells.

Kgp and HRgpA, but not RgpB, are complexes carrying haemagglutinin/adhesin domains (Nakayama *et al.*, 1995; Okamoto *et al.*, 1996), which may be involved in the IL-8-suppressive effects of Kgp and HRgpA. To investigate this possibility, we utilized protein fractions prepared from the culture supernatants of wild-type *P. gingivalis* 33277, *rgpA*- and *rgpB*-defective mutant *P. gingivalis* KDP133, *rgpA*-, *kgp*- and *hagA*-defective mutant *P. gingivalis* KDP137, *rgpA*-, *rgpB*- and *kgp*-defective mutant *P. gingivalis* KDP136, and a mutant KDP160, which carries only the enzymic domain of Kgp transfected into the Rgp/Kgp/adhesin-defective mutant *P. gingivalis* KDP153. KDP161 was used as a control for KDP160. The protein fraction from wild-type *P. gingivalis*, which contains Kgp and HRgpA, and KDP133, which also contains Kgp, significantly inhibited IL-8 secretion, and the protein fraction from KDP136, which contains only the adhesin domain, slightly inhibited the secretion, whereas the partially purified protein from KDP160, which contains the haemagglutinin domain-defective Kgp, and KDP137, which contains RgpB, significantly stimulated IL-8 production (Fig. 6a). These findings suggested that the adhesin domain may be required for the downregulation of IL-8 production by HRgpA and Kgp, although the adhesin domain alone is not sufficient to exert full inhibitory activity. C-terminal adhesin domains (HbR and Hgp44) are responsible for haemagglutination. We examined the effect on HSC-2 cells of rHbR and/or rHgp44 plus RgpB. We did not observe any downregulation of IL-8 production (data not shown). Furthermore, rHbR and rHgp44 marginally but significantly downregulated IL-8 secretion (Fig. 6b). These results suggested that both catalytic

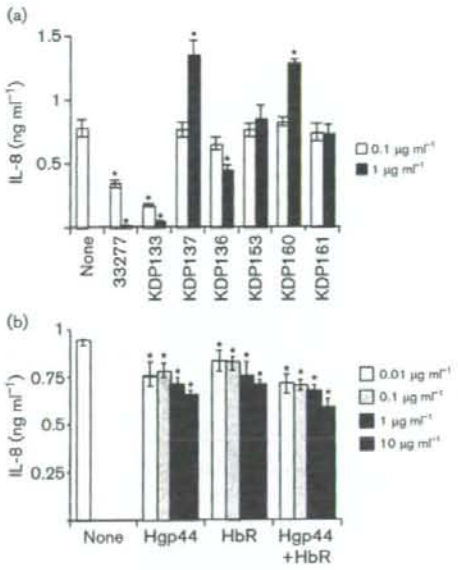

Fig. 6. The haemagglutinin domain is necessary but not sufficient for gingipains to downregulate IL-8 production in human oral epithelial cells. (a) HSC-2 cells were cultured with or without 0.1–1 μg protein fraction ml^{-1} prepared from culture supernatants of wild-type *P. gingivalis* 33277 and mutant *P. gingivalis* strains KDP133, KDP137, KDP136, KDP153, KDP160 and KDP161 for 24 h in triplicate at 37 °C. IL-8 concentration was determined by ELISA and the results shown as means ± SD. (b) HSC-2 cells were cultured with or without 0.01–10 μg recombinant C-terminal adhesin domains (rHbR and/or rHgp44) ml^{-1} for 24 h in triplicate at 37 °C. IL-8 concentrations were determined by ELISA and the results shown as means ± SD. *, $P<0.01$ compared with the respective control.

(enzymic) and haemagglutinin domains exist in the same molecule for Kgp and HrgpA, and exert a powerful downregulatory effect on IL-8 production in human oral epithelial cells.

As gingipains are reported to cleave pro-inflammatory cytokines such as IL-8 (Mikolajczyk-Pawlinska *et al.*, 1998), it may be possible that the enzymic activity of gingipains directly cleaves IL-8. It must be emphasized, however, that in our study clear inhibition of IL-8 mRNA expression by Kgp and HRgpA, but not RgpB, was observed (Fig. 1c). RgpB carrying enzymic activity did not decrease IL-8 production, and mutant KDP137, which carries only the enzymic domain of Kgp, and mutant KDP160, which carries only the enzymic domain of RgpB, also did not decrease IL-8 production (Fig. 6). In addition, we clarified, using RNA interference, that dual regulation of IL-8 production by gingipains involves PAR-1, PAR-2 and the NF-κB signalling pathway (Fig. 4).

Downregulation of IL-8 secretion by gingipains may be a novel mechanism by which *P. gingivalis* evades the host defence system. We demonstrated previously that human oral epithelial cells treated with pro-inflammatory cytokines (IFN-γ, IL-1α or TNF-α) secreted a high level of various pro-inflammatory cytokines, including IL-8, in response to bacterial cell-surface components (Uehara *et al.*, 2002a), although naive human oral epithelial cells in culture did not show enhanced production of pro-inflammatory cytokines upon stimulation with these stimuli (Uehara *et al.*, 2001). Therefore, in the presence of gingipains, oral epithelial cells might be totally devoid of IL-8 production, even upon stimulation with bacterial components. It must be noted that *P. gingivalis* LPS, another putative virulence factor, is suggested to evade recognition by the host via Toll-like receptor 4 (Ogawa *et al.*, 2007). Considering all of these findings, *P. gingivalis* could inhabit periodontal tissues by evading host defence mechanisms and, as a consequence, sustain chronic inflammation.

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Gingipains from *Porphyromonas gingivalis* synergistically induce the production of proinflammatory cytokines through protease-activated receptors with Toll-like receptor and NOD1/2 ligands in human monocytic cells

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Summary

Gingipains (HRgpA, RgpB and Kgp) are cysteine proteinases and virulence factors of *Porphyromonas gingivalis*, the major causative bacterium of periodontal disease. To study synergistic effects of gingipains and signalling via Toll-like receptors (TLRs) and NOD1/2, we investigated effects of a gingipain on the secretion of proinflammatory cytokines from monocytic THP-1 cells in the presence of pathogen-associated molecular patterns (PAMPs). Gingipains stimulated interleukin (IL)-8's secretion from THP-1 cells, which was completely inhibited by proteinase inhibitors of gingipain and increased in the presence of PAMPs. Synergistic effects of gingipains and PAMPs were also seen in the secretion of IL-6 and MCP-1 and reduced to about 50% the secretion of IL-8 from THP-1 cells treated with siRNA targeting either protease-activated receptor (PAR)-1, -2 or -3. PAR agonist peptides mimicked the synergistic effects of gingipains with PAMPs. These results indicate that gingipains stimulate the secretion of cytokines from monocytic cells through the activation of PARs with synergistic effects by PAMPs. This is the first report of synergism of signalling via PARs, and TLRs or NOD1/2. The host defence system against *P. gingivalis* may be triggered through the activation of PARs by gingipains and augmented by PAMPs from this pathogen via TLRs or NOD1/2.

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Introduction

Periodontal disease is chronic gingival inflammation and causes periodontal tissue destruction, loss of alveolar bone, and eventually, tooth loss. *Porphyromonas gingivalis* is the causative pathogen not only for adult periodontitis but also for rapidly progressive periodontitis (Holt and Bramanti, 1991). *P. gingivalis* possesses a number of putative virulence factors, such as lipopolysaccharide (LPS), fimbriae, toxic products of metabolism and proteinases, all of which stimulate host cells to release inflammatory mediators and promote this infectious disease. We have studied virulence activities of two types of cysteine proteinases (Potempa *et al.*, 1995); arginine-specific gingipains (Rgp) of 50 and 95 kDa that cleave peptide bonds specifically at Arg residues (Chen *et al.*, 1992) and a lysine-specific gingipain (Kgp) of 105 kDa that cleaves peptide bonds specifically at Lys residues (Pike *et al.*, 1994). The 95 kDa high molecular mass Rgp (HRgpA) and kgp are complexes of a catalytic domain and a hemagglutinin/adhesion domain, whereas the 50 kDa Rgp (RgpB) lacks the latter domain. Gingipains enhance vascular permeability through activation of the kallikrein/kinin pathway (Imamura *et al.*, 1994; 1995a), disrupt plasma clotting (Scott *et al.*, 1993; Imamura *et al.*, 1995b; 1997), activate components of the complement system (Wingrove *et al.*, 1992) and modify neutrophil functions (Jagels *et al.*, 1996). The conversion of profimbrilin to mature fimbrilin by gingipains is indispensable for the expression of *P. gingivalis* fimbriae (Kadowaki *et al.*, 1998), an important cell surface structure of this bacterium for adhesion, colonization and invasion (Amano, 2007). Furthermore, we have shown that gingipains cleave CD14 on human monocytes (Sugawara *et al.*, 2000) and gingival fibroblasts (Tada *et al.*, 2002), and ICAM-1 on human oral epithelial cells (Tada *et al.*, 2003), inhibiting LPS-elicited defensive responses of these cells to this pathogen and interaction between epithelial

cells and leukocytes, respectively, which help *P. gingivalis* to evade the innate immune responses.

Protease-activated receptors (PARs) are G protein-coupled receptors, characterized by signal transduction triggered through proteolytic cleavage at each N-terminal peptide (Déry *et al.*, 1998; Coughlin, 2000; O'Brien *et al.*, 2001). PAR-1, PAR-3 and PAR-4 are activated mainly by thrombin, while PAR-2 is activated by trypsin and mast cell tryptase as well as the coagulation factors VIIa and Xa but not by thrombin (Déry *et al.*, 1998; Coughlin, 2000; O'Brien *et al.*, 2001). We added neutrophil serine proteinase 3 (PR3) as another activator of PAR-2 on human oral epithelial cells and human gingival fibroblasts (Uehara *et al.*, 2002; 2003). PARs are expressed on a wide variety of cell types, and suggested to play important roles in pathophysiological processes, such as growth, development, inflammation, tissue repair and pain (Déry *et al.*, 1998; Coughlin, 2000; O'Brien *et al.*, 2001). RgpB activated PAR-2 on human neutrophils (Lourbakos *et al.*, 1998), and induced interleukin (IL)-6's secretion by activating PAR-1 and PAR-2 on human oral epithelial KB cells (Lourbakos *et al.*, 2001a) and platelet aggregation via PAR-1 and PAR-4 (Lourbakos *et al.*, 2001b). Furthermore, RgpB induced the release of neuropeptide from dental pulp cells via PAR-2 signalling (Tancharoen *et al.*, 2005). Recently, we revealed that Rgps (HRgpA and RgpB) stimulated production of hepatocyte growth factor (HGF) through PAR-1 and PAR-2 in human gingival fibroblasts (Uehara *et al.*, 2005a), which may be associated with both inflammatory and reparative processes in periodontal tissues. Therefore, PARs are important molecules that mediate gingipains' stimulatory effects on cells.

The innate immune system recognizes microorganisms through a series of pattern recognition receptors that are highly conserved and bind specifically to common motifs, designated pathogen-associated molecular patterns (PAMPs), present in microorganisms but not in eukaryotes. Representative PAMPs are the lipid A moiety of LPS, lipopeptides, peptidoglycans (PGNs), bacterial DNA, viral double-stranded and single-stranded RNA. Several studies have demonstrated that in mammals, these PAMPs are recognized specifically by the respective Toll-like receptor (TLR) (Akira *et al.*, 2006). In addition, the NOD-like receptor (NLR) family were demonstrated to be intracellular receptors for a partial structure of PGN; NOD1 and NOD2 recognize diaminopimelic acid (DAP) containing a peptide moiety and muramyl peptide respectively (Fritz *et al.*, 2006).

We revealed that a combination of chemically synthesized TLR agonists with muramyldipeptide (MDP) or DAP-containing desmuramylpeptides synergistically induced production of IL-8 in a NOD2- or NOD1-dependent manner, respectively, in human monocytic THP-1 cells in culture (Uehara *et al.*, 2005b). Furthermore, we recently reported that anti-PR3 Abs primes human monocytic cells through PAR-2 for TLR- and NOD-dependent enhanced cell activation (Uehara *et al.*, 2007). These results suggest an interaction of signalling triggered PARs and TLRs or NODs. Therefore, we investigated the possible synergistic effect of a PAR agonist peptide (AP) (PAR-1AP, -2AP or -3AP) and a TLR/NLR ligand agonist on the production of IL-8 in human monocytic cells. Then, we examined whether gingipains as PAR agonists exert a synergistic effect in combination with a TLR/NLR agonist to present a novel activity of gingipains.

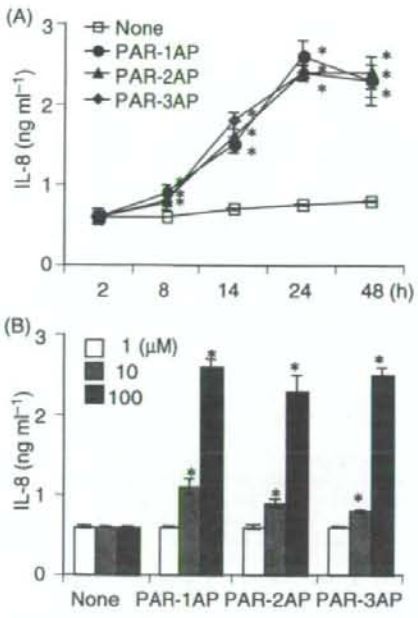

Fig. 1. The time-course and dose response of PAR-AP-induced IL-8 production.
A. THP-1 cells were incubated with 100 μM of PAR-1AP, PAR-2AP or PAR-3AP for a range of different time periods.
B. THP-1 cells were incubated with one of the PAR-APs at various concentrations for 24 h. The concentrations of IL-8 released into supernatants were measured by ELISA and values are the means ± SD for triplicate assays. *$P < 0.01$ versus the culture medium alone. Results representative of three different experiments are shown.

Results

Increase of IL-8 secretion from cultured human monocytic THP-1 cells by PAR-APs

First, we investigated the effect of PAR signalling (PAR-1, -2 and -3) on the production of IL-8 by THP-1 cells. PAR-APs significantly promoted IL-8's secretion from the cells over an incubation period of 8–24 h, after which there appeared to be a slight decrease (Fig. 1A). PAR-APs

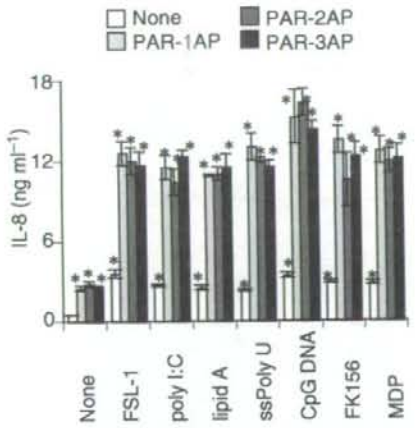

Fig. 2. Synergistic effect of PAR-APs and synthetic TLR and NOD ligands on IL-8 secretion from cultured THP-1 cells. THP-1 cells were stimulated with 100 μM of a PAR-AP in the presence of either FSL-1 (1 nM), poly I:C (1 μg ml^{-1}), lipid A (10 ng ml^{-1}), ssPoly U (10 μg ml^{-1}), CpG DNA (1 μM), FK156 (100 μg ml^{-1}) or MDP (100 μg ml^{-1}) for 24 h. Concentrations of IL-8 in the culture supernatants were measured by ELISA, and values are means ± SD for triplicate assays. *$P < 0.01$ versus the culture medium alone and the respective control respectively. Results representative of three different experiments are shown.

increased the amount of IL-8 secreted in a dose-dependent manner from 10 μM to at least 100 μM (Fig. 1B). The secretion was augmented about fourfold by PAR-APs at 100 μM and the three types of PAR agonists had similar stimulatory effects.

Synergistic effect of PAR-APs and synthetic TLR or NOD ligands on secretion of IL-8 from cultured THP-1 cells

As reported (Uehara *et al.*, 2005b), synthetic PAMPs induced the secretion of IL-8 in THP-1 cells in a dose-dependent manner (data not shown). To elucidate the possible synergistic effects of PAR-APs (PAR-1AP, -2AP and -3AP) and various TLR or NOD agonists, we examined IL-8's secretion from THP-1 cells in the presence of both a PAR-AP and a synthetic PAMP. Clear synergistic effects were observed with all combinations of PAR-APs and PAMPs and the secretion of IL-8 evoked by a PAR-AP increased about four- to fivefold in the presence of any of the PAMPs (Fig. 2).

Increase in secretion of IL-8 caused by gingipains in cultured human monocytic THP-1 cells

We demonstrated that Rgps activate PAR-1 and PAR-2, stimulating the production of hepatocyte growth factor in cultured human gingival fibroblasts (Uehara *et al.*, 2005a). We therefore studied the effects of gingipains (Kgp, HRgpA and RgpB), as PAR agonists, on the secretion of proinflammatory cytokines from THP-1 cells. Gingipains increased production of IL-8 in a time-dependent manner (Fig. 3A). A significant increase in secretion was observed over an incubation period of 14–24 h, and Kgp and HRgpA were more effective than RgpB (Fig. 3A).

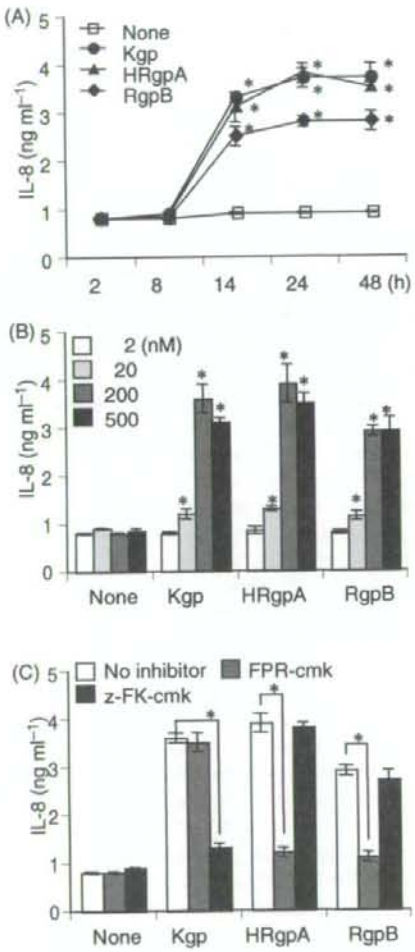

Fig. 3. Effects of gingipains on IL-8 production by THP-1 cells.
A. THP-1 cells were incubated with 200 nM of gingipains for various periods.
B. THP-1 cells were incubated with various concentrations of gingipains for 24 h.
C. THP-1 cells were incubated with gingipains, pretreated with 10 μM of FPR-cmk or z-FK-cmk for 15 min at 37°C, for 24 h. The solution for gingipain activation was used as a control. The concentrations of IL-8 in the supernatant were measured by ELISA and values are the means ± SD for triplicate assays. *$P < 0.01$ versus the culture medium alone or respective counterpart. Results representative of three different experiments are shown.

Gingipains augmented IL-8's secretion in a dose-dependent manner starting at a gingipain concentration of 20 nM (Fig. 3B). FPR-cmk and z-FK-cmk, inhibitors specific to Rgps and Kgp respectively (Potempa *et al.*, 1997), completely inhibited the gingipain-induced secretion of IL-8, indicating its dependency on the enzymatic activities (Fig. 3C).

Synergistic effect of gingipains and synthetic TLR or NOD ligands on secretion of proinflammatory cytokines from THP-1 cells

Next, we examined the synergistic effects of gingipains and the synthetic PAMPs on cytokine production. Among PAMPs, Poly I:C and CpG DNA induced definite secretion of the three cytokines in the absence of gingipains (Fig. 4). Similar to PAR-APs, the three gingipains exhibited synergistic effects with these PAMPs on IL-8's secretion from THP-1 cells, as was seen for the secretion of IL-6, and monocyte chemoattractant protein (MCP)-1 and HRgpA were most effective in combination with any PAMP, except with FK156 in the secretion (Fig. 4).

Synergistic effect of gingipains and synthetic TLR or NOD ligands on secretion of proinflammatory cytokines from human peripheral blood mononuclear cells (PBMCs)

Next, we examined whether similar synergistic effects of gingipains and synthetic TLR or NOD ligands were observed in human PBMCs. Consistent with the results for THP-1 cells, the gingipains exhibited synergistic effects with these PAMPs on the secretion of IL-8, IL-6 and MCP-1 from PBMCs (Fig. 5).

Involvement of PARs in synergistic effects of gingipains on IL-8 secretion from THP-1 cells

To confirm the involvement of PARs in the synergistic effects of gingipains and PAMPs, TLR agonists or NOD agonists, we used short-interfering RNA (siRNA) to block the expression of PAR-1, -2 or -3. Protein levels of PAR-1, -2 and -3 in the cells treated with the siRNA were suppressed about 80% (Fig. 6A). As shown in Fig. 6B, synergistic effects of gingipains and synthetic PAMPs on the secretion of IL-8 were significantly inhibited to about 50% in all of the PAR-silenced THP-1 cells, but not in Lamin-silenced THP-1 cells, irrespective of PAMPs. These results clearly indicate PAR-1, -2 and -3 to be critical for the synergistic effects of gingipains and TLR or NOD agonists.

Discussion

Porphyromonas gingivalis cysteine proteinases, Rgps and Kgp, synergistically increase the secretion of pro-

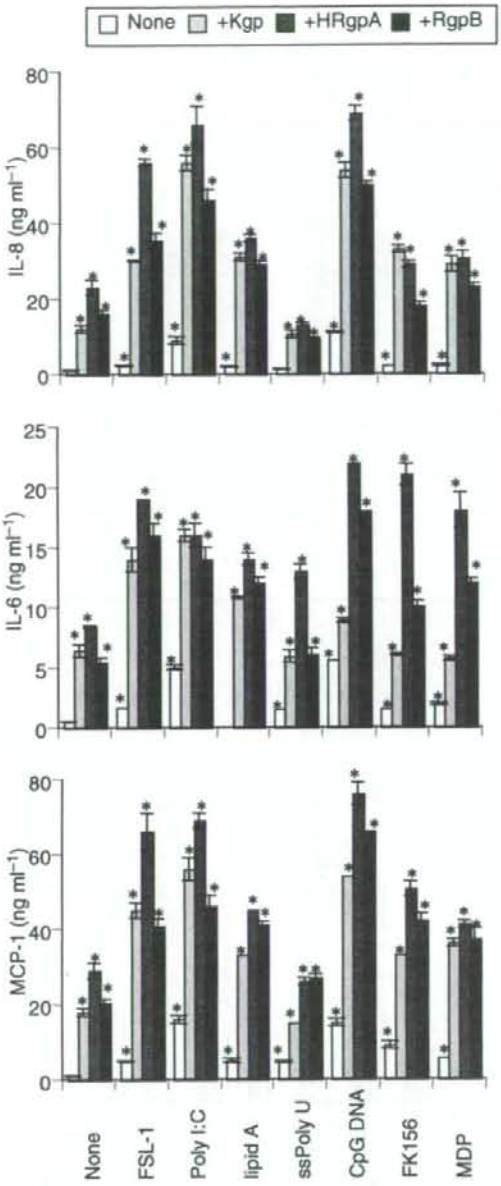

Fig. 4. Synergistic effects of gingipains and synthetic TLR or NOD ligands on secretion of proinflammatory cytokines in cultured THP-1 cells. THP-1 cells were incubated with 200 nM of Kgp, HRgpA, or RgpB in the presence of either of FSL-1 (1 nM), poly I:C (1 μg ml^{-1}), lipid A (10 ng ml^{-1}), ssPoly U (10 μg ml^{-1}), CpG DNA (1 μM), FK156 (100 μg ml^{-1}) or MDP (100 μg ml^{-1}) for 24 h. Concentrations of IL-8, IL-6 and MCP-1 in the culture supernatants were measured by ELISA, and values are the means ± SD for triplicate assays. *$P < 0.01$ versus the culture medium alone and the respective control. Results representative of four different experiments are shown.