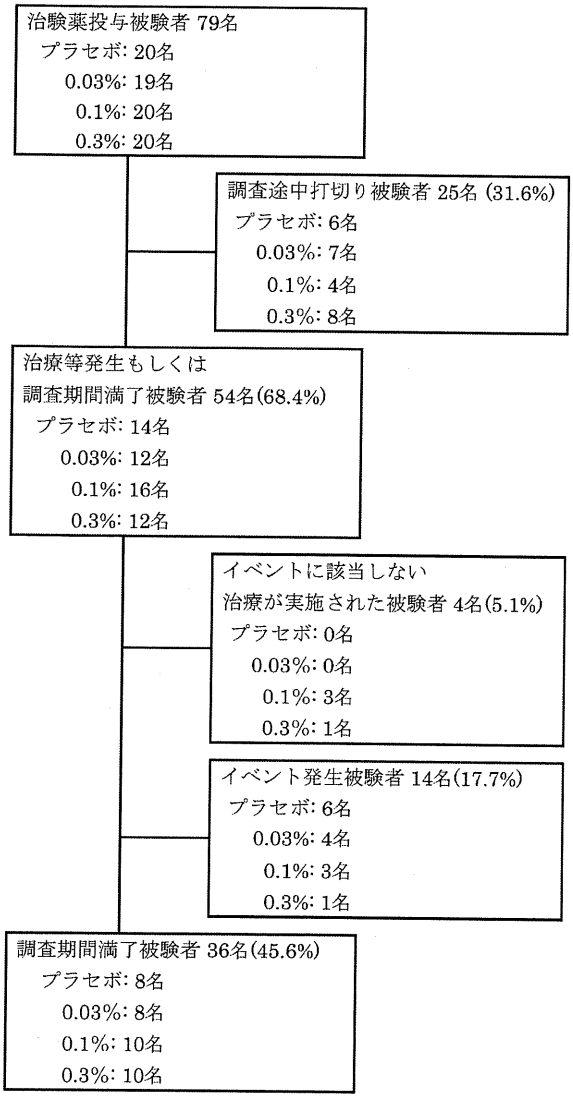

図1　被験者構成

生したか否かを十分に判断できない調査途中打ち切り被験者であった。残りの54名の中で，イベント発生被験者は14名，イベントに該当しない治療等（治療計画上の積極的な抜歯，根尖性歯周炎のための歯根端切除術等）により打ち切りとなった被験者は4名，そして，調査実施日までイベントの発生やイベントに該当しない治療の実施がなかったことが診療情報から確認できた調査期間満了被験者は36名であった（図1）。

2．各群の被験者，被験歯および試験部位の特性と観察期間

KCB-1D探索的試験[5]参加時の被験者の性別や年齢，被験歯の歯種および試験部位の骨吸収等の臨床所見に特記すべき群間の偏りは認められていない。そして，治験薬投与日から最後に被験歯を観察した日までの期間も，全ての群で同程度であった（表1）。

表1　各群の治験薬投与日から最後に被験歯を観察した日までの期間

	中央値(日)	範囲(最小－最大)
プラセボ投与群(20名)	2,887	714－3,143
0.03%FGF－2投与群(19名)	2,879	263－3,093
0.1%FGF－2投与群(20名)	2,886	168－3,150
0.3%FGF－2投与群(20名)	2,868	249－3,122

3．イベント

各群で発生したイベントの内容と発生数を表2に示す。イベントが発生した14名の中で，積極的な介入をした非外科的歯周治療の発生数が7名と最も多かった。続いて，歯周組織再生療法（EMD，GTR等）が3名と多く，抜歯と歯周組織再生療法を除く歯周外科手術がそれぞれ2名であった。その他，歯周炎の進行が原因となって生じた事象は全ての群で発生しなかった。また，各群のイベントの発生数は，プラセボ群6名，0.03%群4名，0.1%群3名，0.3%群1名であった（図1および表2）。

4．生存時間解析

治験薬投与日（0日目）からの経過日数に対するイベント未発生率を表すKaplan-Meier曲線を図2に示す。生存時間解析の結果，0.3% FGF-2投与群はプラセボ群に比べてイベント発生までの期間の有意な延長が認められた(一般化Wilcoxon検定：$p=0.0345$)。すなわち，0.3%FGF-2投与群では投与後2800日(約7.6年)程度で1名にイベントが発生し，観察期間の最終的なイベント未発生率は約80%であった。これに対してプラセボ群では，投与後2000日(約5.4年)まではイベント未発生率80%を維持していたものの，それ以降にイベント発生が増加し，最終的なイベント未発生率は約60%であった（図2）。

5．安全性

本研究の観察期間中に，被験歯に異常な歯周組織の治癒（歯肉増殖等）が疑われる所見は認められなかった。

表2　発生したイベントと各群における発生数

(1)抜歯 (治験薬投与部位を有する歯根の抜去を含む)	全体 2名 (2.5%) プラセボ: 1名 0.03%: 0名 0.1%: 1名 0.3%: 0名
(2)歯周組織再生療法 (EMD, GTR等)	全体 3名 (3.8%) プラセボ: 2名 0.03%: 1名 0.1%: 0名 0.3%: 0名
(3)歯周組織再生療法を除く歯周外科手術	全体 2名 (2.5%) プラセボ: 0名 0.03%: 0名 0.1%: 2名 0.3%: 0名
(4)積極的な介入をした非外科的歯周治療 (歯肉縁下の処置を目的としたスケーリング・ルートプレーニング，局所抗菌薬投与等)	全体 7名 (8.9%) プラセボ: 3名 0.03%: 3名 0.1%: 0名 0.3%: 1名
(5)その他，歯周炎の進行が原因となって生じた事象(逆行性歯髄炎等)	全体 0名 (0%) プラセボ: 0名 0.03%: 0名 0.1%: 0名 0.3%: 0名
合計	全体14名 (17.7%) プラセボ: 6名 0.03%: 4名 0.1%: 3名 0.3%: 1名

考　察

現在，進行した歯周炎に対する歯周外科処置としては，組織付着療法に分類されるフラップ手術が標準的治療法として施行されている。フラップ手術は，歯根面および歯周ポケット内部に蓄積した細菌および細菌由来病原物質を汚染セメント質とともに除去することが可能であり，術後には，炎症の軽減，臨床的アタッチメントの獲得，歯周ポケットの減少が認められる[7]。しかしながら，フラップ手術単独では，本来の歯周組織に見られる歯槽骨，歯根膜およびセメント質の新生を伴う線維性付着はわずかしか形成されず，歯周組織の再生はほとんど期待できない。また，臨床的アタッチメントの獲得も長い上皮性付着の形成によることが多く[8]，長期的には再度の付着喪失を伴う歯周炎の再発が懸念される。そのため，フラップ手術等の従来の歯周外科処置では達成できない歯周組織の再生に対する臨床現場の期待は非常に大きく，盛んに研究開発が行われてきた。そして現在，歯周組織再生誘導法(GTR 法) およびエナメルマトリックスタンパク(EMD) を用いた歯周組織再生療法が我国で臨床適応されており，米国では血小板由来増殖因子(PDGF-BB)と骨補填材である多孔性リン酸三カルシウム(β-tricalcium phosphate; β-TCP) との合剤がヒト型リコンビナントサイトカインを用いた歯周組織誘導材料として登場している[9]。しかしながら，これらの方法は，治療成績が術者の技術レベルに大きく左右されたり，動物由来製剤であったりといった問題点から，重度歯周炎に対する標準的治療法となり得る歯周組織再生誘導薬の開発が強く求められている。そこで，我々は，様々な細胞に対する生理活性を有するFGF-2 を，歯周組織再生誘導薬の有力な候補の一つと考え，その歯周組織誘導能の動物実験[2-4]による確認を経て，3，2 壁性歯槽骨欠損を有する歯周炎患者を対象とした第 II 相臨床治験[5,6]を展開してきた。その結果，FGF-2 投与 36 週後において，通常のフラップ手術と同程度の付着を獲得するとともに有意な歯槽骨新生がもたらされたことから，FGF-2 の歯周組織再生誘導薬としての有用性が強く示唆されている。そこで，本研究では，歯周組織再生療法の真の目的が長期的な歯の保存であることを考慮し，投与 36 週後において歯周組織再生誘導薬としての有用性が確認された FGF-2 のさらに長期的な効果と安全性を検討するため，第 II 相臨床治験の中でも臨床治験終了後の経過期間が約 8 年と長期に及ぶ KCB-1D 探索的試験[5]に参加した被験者を対象として，フラップ手術時に各種濃度の FGF-2 あるいはプラセボを投与した歯の長期経過を調査した。

歯周組織の再生を歯槽骨，セメント質，歯根膜などの再生と定義[10]すれば，これらの組織の再生を直接かつ明確に確認する唯一の方法は組織学的評価である。しかしながら，ヒトでの組織学的な評価は倫理的な観点から困難を極めるため，一般的に臨床試験では，規格エックス線写真による歯槽骨レベルの評価や臨床的アタッチメントレベル (CAL) の測定が歯周組織再生を評価する代替評価項目として利用される[10]。しかしながら，数年を超える長期の臨床経過の追跡では，その間に咬耗・摩耗や修復処置による歯冠形態の変化や歯の移動が生じるケースが多く，X 線検査の規格化や一定の基準点を設定した CAL の測定は，実際には不可能な場合が多い。そこで，本研究では，一連の動的な歯周治療を受けサポーティブペリオドンタルセラピィを受けている被験者では，歯周炎の治療経過が悪

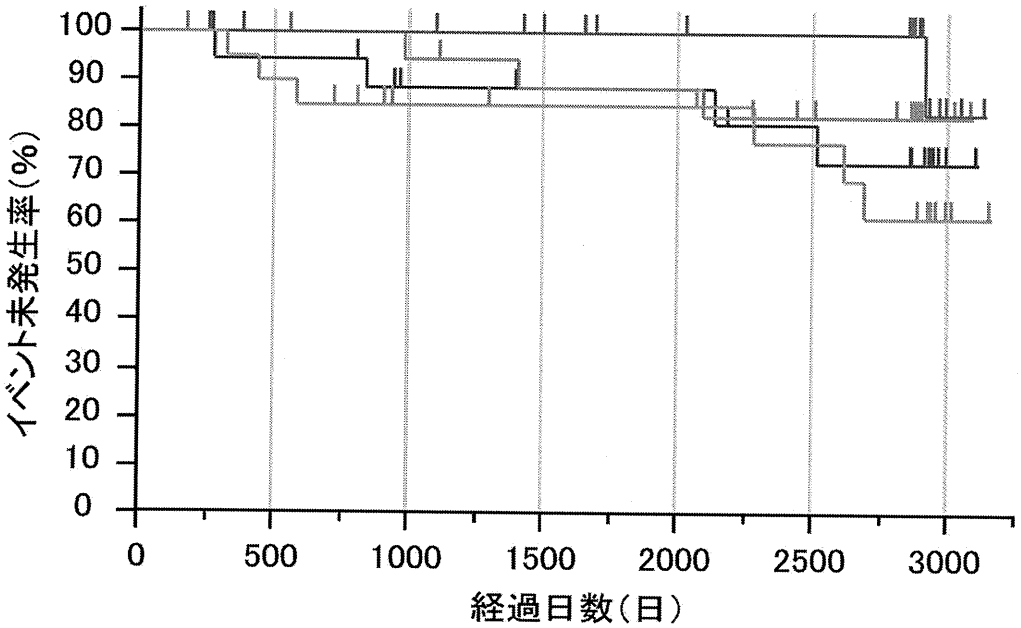

図2　イベント未発生率を表すKaplan-Meier曲線

赤：0.3%FGF-2投与群，緑：0.1%FGF-2投与群，青：0.03%FGF-2投与群，桃：プラセボ投与群。各群のKaplan-Meier曲線上のヒゲは打ち切りを表す。生存時間解析の結果，0.3%FGF-2投与群はプラセボ群に比べてイベント発生までの期間の有意な延長が認められた（一般化Wilcoxon検定：$p=0.0345$）。

く抜歯に至るケースでも抜歯に至る前に歯周外科的処置や歯周ポケットに対する非外科的な処置が通常行われることにヒントを得て，表2に示した(1) 抜歯，(2) 歯周組織再生療法，(3) 歯周組織再生療法を除く歯周外科手術，(4) 積極的な介入をした非外科的歯周治療 (5) その他，歯周炎の進行が原因となって生じた事象をイベントとして，FGF-2投与の長期的効果を判定する評価指標とした。

本研究では，転院等によって診療情報の一部が得らなかったため観察期間に治療等が発生したか否かを十分判断できなかった被験者は全被験者（79名）の約30%（25名）であった（図1）。しかしながら，KCB-1D探索的試験[5]で割り付けられた被験者の背景情報および調査途中打切り被験者数に大きな群間の偏りがなく，本研究における各群の観察期間が各群で同程度であったことから（表1），各群の被験者の一般的特性が結果に大きな影響を与えることはなかったと考えられる。

観察期間における各群のイベントの発生数は，プラセボ群6名，0.03%群4名，0.1%群3名，0.3%群1名と，高濃度のFGF-2を投与した群ほど低かった（図1および表2）。さらに，生存時間解析の結果においても，0.3% FGF-2投与群がフラップ手術単独施行に相当するプラセボ群に対して有意にイベント発生までの時間を延長させることが示され，0.3%FGF-2投与が，歯周炎の再発・悪化のリスクを長期的に低減することが示唆された（図2）。

本研究で被験歯に歯周炎の悪化による抜歯を認めたのは約2.5%(2歯)で，当初予想した通り非常に少なかった。Palcanis[11]はフラップ手術を含む歯周外科処置が歯の長期的な保存に与える影響を総説として報告している。この総説によると，Ramfjordら[12]は，歯周外科処置を行った1800歯の5年間の経過観察によって22歯(約1.2%)が抜去されていたことを報告しているが，その他の多くの長期観察では抜歯はほとんど認められていない。また，5年未満の観察では，フラップ手術後の臨床的アタッチメントの喪失は多くの場合1mm未満であり，5年程度の経過観察ではフラップ手術とFGF-2投与群との術後のイベント発生に大きな違いを認めなかったという本研究の結果と合致する。今回対象とした被験者が参加したKCB-1D探索的試験[5]の結果を受け2005年～2007年に実施されたKCB-1D用量反応試験[6]における同様の観察では，観察期間が約5年間と本研究の観察期間の8年より短く，イベントの発生に統計学的に有意な群間差を認めていない（結果未発表）。以上の結果から，治療に

よる長期的予後の違いを検討するには本研究と同程度か，それ以上の観察期間を要するものと考えられる。今後，より多くの被験者が参加した臨床治験を対象とした同様の観察研究の検討が望まれる。

本研究の結果も含め，これまでの研究からフラップ手術後に認められる長い上皮性の付着による治癒形態は，術後5年程度であれば維持されることが示唆されることから，フラップ手術が患者に与えるメリットは少なくない。しかしながら，それ以降の長期的な歯の予後には，FGF-2投与により歯槽骨の再生および結合性付着の再構築を図ることが有益であることが本研究で示された。世界初の歯周組織再生薬として市場に出たFGF-2が，歯周炎患者の歯の喪失を防ぎ，口腔の働きが支えるQOLの向上に寄与することが期待される。

結　論

FGF-2を用いた歯周組織再生療法臨床治験の施行後約8年間の観察で，0.3% FGF-2投与がフラップ手術単独と比較して再治療等のイベント発生までの期間を延長させることが示された。また，フラップ手術単独群のイベントの発生率は5年以降に増加する傾向が認められ，治療による長期的予後の違いを検討するには本研究と同程度かそれ以上の観察期間を要することが示唆された。また，本研究の観察期間を含めて安全性に関する問題は認めなかった。

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Osteoinductive and anti-inflammatory effect of royal jelly on periodontal ligament cells

Manabu YANAGITA, Yuko KOJIMA, Kenta MORI, Satoru YAMADA, and Shinya MURAKAMI
Department of Periodontology, Division of Oral Biology and Disease Control, Osaka University Graduate School of Dentistry, Osaka 565-0871, Japan

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ABSTRACT

Royal jelly (RJ) has been reported to possess several physiological and pharmacological properties such as the ability to prevent osteoporosis in rats and anti-inflammatory effects. We hypothesized that RJ could have beneficial effects on the prevention or treatment of periodontal diseases, which are chronic inflammatory diseases caused by bacterial infection that result in resorption of the tooth-supporting bone. We assessed the effect of RJ on mineralization in mouse periodontal ligament cell clone 22 (MPDL22 cells), which are of an osteogenic and cementogenic lineage. The mRNA expression of osteopontin, osteocalcin and osterix, and mineralized nodule formation were significantly enhanced in RJ-treated MPDL22 cells. In addition, we investigated the effects of RJ on the production of inflammatory cytokines from MPDL22 cells stimulated with lipopolysaccharide (LPS) of *Porphyromonas gingivalis*, a periodontopathic bacterium. RJ suppressed LPS-induced interleukin-6 and CXC chemokine ligand 10 production from MPDL22 cells. Furthermore, RJ suppressed the expression of CD54 in MPDL22 cells: CD54 is the adhesion molecule involved in the accumulation of leukocytes in periodontal lesions. These findings suggest that the osteoinductive and anti-inflammatory effects of RJ can provide benefits for the treatment and prevention of periodontal diseases.

Periodontal disease is a bacterial biofilm-induced chronic inflammatory disease characterized by the destruction of periodontal tissues including the periodontal ligament (PDL), cementum, gingiva and alveolar bone (10, 24). Among these, PDL, which is located between the alveolar bone and cementum of the tooth, plays crucial roles in the homeostasis and repair/regeneration of periodontal tissues. PDL has been revealed to possess multipotential mesenchymal stem cells that can differentiate into osteoblasts and cementoblasts which can create alveolar bone and cementum (21), respectively, and to express bone-related markers (2, 18). Furthermore, several lines of evidence have shown that PDL cells produce inflammatory cytokines and chemokines, such as interleukin (IL)-6 and IL-8 in response to lipopolysaccharide (LPS) of *Porphyromonas gingivalis* (*P. gingivalis*), one of the causative black-pigmented Gram-negative anaerobes, responsible for the development of chronic inflammation in the periodontium (27). Thus, PDL cells are involved in the repair and maintenance of the periodontium, as well as in inflammation.

Royal jelly (RJ) is produced in the hypopharyngeal and mandibular glands of worker honeybees (*Apis mellifera*) and is a necessary food for the growth of the queen honeybee. RJ consists of proteins (18%), sugars (15%), lipids (3–6%) and water (50–60%). RJ also contains vitamins, amino acids and minerals (17). Interestingly, RJ has been shown to exhibit

Address correspondence to: Dr. Shinya Murakami, PhD, D.D.S., Department of Periodontology, Division of Oral Biology and Disease Control, Osaka University Graduate School of Dentistry, Yamadaoka 1-8, Suita, Osaka 565-0871, Japan
Tel: +81-6-6879-2930, Fax: +81-6-6879-2934
E-mail: ipshinya@dent.osaka-u.ac.jp

several biological activities, including anti-hypercholesterolemic (25), anti-tumor (3), anti-microbial (4), anti-allergic (20), anti-inflammatory properties (11), and vasodilative and hypotensive activities (22). Recent studies have shown that RJ has weak estrogenic effects and prevents osteoporosis in ovariectomized rats (7, 15). Like osteoporosis, periodontal diseases are typically accompanied by bone resorption. In fact, the positive association between osteoporosis and periodontitis has been suggested (13). We hypothesized that administration of RJ in the daily diet or local application of RJ to periodontal tissue might prevent periodontal disease.

In the present study, we investigated the effects of RJ on osteogenic mineralized nodule formation using mouse PDL (MPDL) cells and on the secretion of IL-6 as inflammatory cytokine and CXC chemokine ligand 10 (CXCL10), chemokine suggested to be involved in alveolar bone destruction in periodontal diseases lesion (9), from LPS-stimulated MPDL cells.

MATERIALS AND METHODS

Reagent. Dried powders of raw RJ originating from China were supplied by Yamada Apiculture Center, Inc. (Okayama, Japan). LPS of *P. gingivalis* was purchased from InvivoGen (San Diego, CA, USA).

Culture of MPDL. In a previous work, we established an MPDL clone cell line, MPDL22, isolated from the PDL tissue of the molar teeth extracted from 2.5-week-old BALB/c mice (26). MPDL22 cells were maintained in α-MEM (Nikken, Kyoto, Japan) supplemented with 10% fetal calf serum (FCS; JRH Biosciences, Lenexa, KS, USA) and 100 ng/mL fibroblast growth factor-2 (FGF-2; Kaken, Kyoto, Japan) (26). When the cells reached confluence, we replaced the culture medium (α-MEM supplemented with 10% FCS and FGF-2) with the mineralization medium (α-MEM supplemented with 10% FCS, 10 mM β-glycerophosphate, and 50 mg/mL ascorbic acid).

Proliferation assay. MPDL22 cells (5×10^3 cells/well) were incubated in 96-well plates in α-MEM containing 1% FCS in the presence of RJ or FGF-2 (100 ng/mL) for 48 h. Cell proliferation was measured using the nonradioactive colorimetric assay WST-1 assay (Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer's instructions. The OD450/650 was measured after 2 h on a microplate reader (Bio-Rad, Hercules, CA).

Real-time PCR for osteopontin, osteocalcin and osterix mRNA. RNA samples were obtained from MPDL22 cells cultured in mineralization medium for 3, 5, and 7 days in the presence or absence of RJ. Total RNA extract (0.4 mg) was reverse-transcribed using a High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Foster City, CA, USA) to generate single-stranded cDNA. PCR reactions were carried out using the ABI 7300 Fast Real-Time PCR System (Applied Biosystems) with Power SYBR^R Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. All reactions were run in triplicate. The primer sequences used for real-time PCR were as follows; osteopontin, (sense) 5'-TAC GAC CAT GAG ATT GGC AGT GA-3', (antisense) 5'-TAT AGG ATC TGG GTG CAG GCT GTA A-3'; osteocalcin, (sense) 5'-AGC AGC TTG GCC CAG ACC TA-3', (antisense) 5'-TAG CGC CGG AGT CTG TTC ACT AC-3'; osterix, (sense) 5'-CGC ATC TGA AAG CCC ACT TG-3', (antisense) 5'-CAG CTC GTC AGA GCG AGT GAA-3'.

Mineralization assay. Calcified nodules were stained by an alizarin red staining method. Cell layers were washed twice with phosphate-buffered saline (PBS) and then fixed in dehydrated ethanol. After fixation, the cell layers were stained with 1% alizarin red in 0.1% NH_4OH (pH 6.3–6.5) for 5 min. The culture dishes were then washed with water and observed, digitized and analyzed using WinRoof software (Mitani Corporation, Fukui, Japan).

Measurement of CXCL10 and IL-6 production. MPDL22 cells were seeded in 24-well culture dishes with α-MEM supplemented with 10% FCS and 100 ng/mL FGF-2. When the cells reached 60–70% confluence, quiescent was induced by replacing the medium with serum-free α-MEM for 24 h, and then the medium was replaced with α-MEM supplemented with 1% FCS with or without *P. gingivalis* LPS and RJ. The supernatants from the MPDL22 cultures were collected after 48 h. The concentrations of CXCL10 and IL-6 in the supernatants were measured by ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. In some experiments, MPDL22 cells were cultured for 1, 2, 4, or 6 h in the presence of RJ prior to LPS stimulation.

Flow cytometric acquisition. MPDL22 cells were seeded in 24-well culture dishes with α-MEM supplemented with 10% FCS and 100 ng/mL FGF-2.

When the cells reached 60–70% confluence, quiescent was induced by replacing the medium with serum-free α-MEM for 24 h, and then the medium was replaced with α-MEM supplemented with 1% FCS with or without *P. gingivalis* LPS and RJ. After 48 h, the cells were washed with PBS harvested by incubation with Cell Dissociation Solution (Sigma-Aldrich). Cells were washed with PBS and stained with phycoerythrin-conjugated rat anti-mouse CD54 antibody (eBiosciences, San Diego, CA, USA) for 30 min. The cells were washed twice and data were acquired using FACSCalibur (BD Biosciences, San Jose, CA, USA). Analyses of viable cells were performed using CELLQuestTM software (BD Biosciences).

Statistical analysis. Results were analyzed for statistical significance using analysis of variance with the Bonferroni test. Differences were considered significant at P values less than 0.05. The mRNA expression levels were normalized against the expression levels of hypoxanthine phosphoribosyltransferase 1 (HPRT).

RESULTS

Effect of RJ on MPDL22 proliferation

To investigate the effect of RJ on the proliferation response of MPDL cells, cells were cultured for 48 h in a medium containing 1% FCS with or without RJ. As previous studies have shown that FGF-2 induced the proliferation of PDL cells (28), FGF-2 at a concentration of 100 ng/mL, which was the most optimal concentration for MPDL proliferation in preliminary experiment, was used as a positive control of this assay. Exposure to 0.004–0.5 mg/mL RJ did not induce significant proliferative responses in MPDL22 cells (Fig. 1).

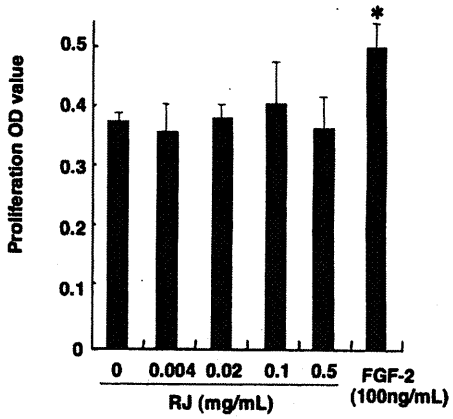

Fig. 1 Effect of RJ on proliferation of MPDL22 cells. MPDL22 cells were cultured with various doses of RJ or FGF-2 (100 ng/mL) for two days. RJ did not induce significant proliferative responses in MPDL22 cells. Values are the means ± SD of four assays. $^*P < 0.05$ compared with medium only.

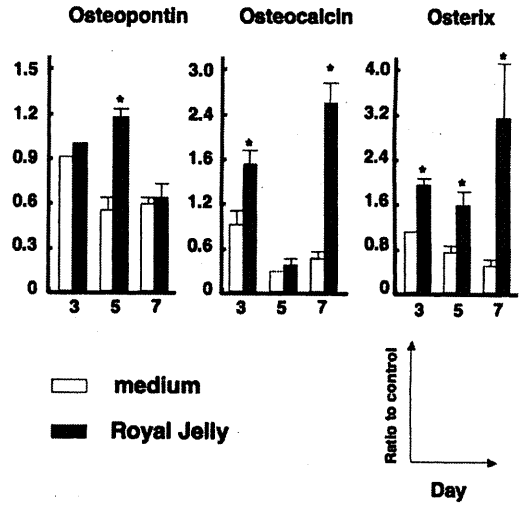

Fig. 2 Analysis of mRNA expression of osteopontin, osteocalcin and osterix in MPDL22 cells. RNA samples were obtained from MPDL22 cells at 3, 5 and 7 days after RJ treatment (0.5 mg/mL). The relative expression of each gene was normalized to the expression levels of HPRT, and the expression of osteopontin, osteocalcin and osterix in MPDL22 cells without RJ was taken as 1.0. RJ significantly enhanced mRNA expression of osteopontin, osteocalcin and osteorix in MPDL22 cells. Values are the means ± SD of three or four assays. $^*P < 0.05$ compared with non-treated cells.

Effects of RJ on osteopontin, osteocalcin and osterix gene expression in MPDL22 cells

We examined the effects of RJ (0.5 mg/mL) on gene expression of extracellular matrices such as osteopontin and osteocalcin, and osteoblastic transcription factor, osterix (Fig. 2). RJ-treated-MPDL22 cells showed significantly enhanced mRNA expression of osteopontin (day 5), osteocalcin (day 3, 7) and osterix (day 3, 5, 7). Although RJ enhanced osterix gene expression at all points we investigated, there was the difference of gene expression pattern between osteopontin and osteocalcin. Whereas osteopontin expression was upregulated with peak at day 5, osteocalcin had two peaks of mRNA expression at day 3 and day 7. The mechanism of suppression of osteocalcin expression at day 5 was unclear, however enhanced mRNA expression of osteocalcin at day 7 suggested emerging mature osteoblastic cells in the presence of RJ.

Effects of RJ on mineralization in MPDL22 cells

We cultured MPDL22 cells with or without RJ in mineralization medium and then examined mineralized nodule formation on day 12. We exposed confluent MPDL22 cells to RJ at various concentrations. As shown in Fig. 3, RJ significantly enhanced alizarin red staining intensity, and in particular, addition of 0.1 mg/mL RJ was most effective.

Effects of RJ on anti-inflammatory responses in MPDL22 cells

To investigate the effects of RJ on the LPS-stimulated CXCL10 production, IL-6 secretion and CD54 expression, MPDL22 cells were pretreated with the indicated concentration of RJ for 1 h and stimulated with LPS for 48 h. As shown in Fig. 4A, a dose-dependent suppressive effect of RJ on CXCL10 production by MPDL22 cells was observed. Although more than half of reduction of CXCL10 production in the prersence of RJ was shown, an approximately 30% reduction in IL-6 production was observed at each concentration of RJ investigated (Fig. 4B). The most effective dose of RJ for inhibition of IL-6 production was 0.1 mg/mL. CXCL10 and IL-6 inhibition was most efficient when RJ treatment occurred 6 h before LPS stimulation (Fig. 4C and D).

A previous study demonstrated that bacterial components induced a cell surface molecule CD54 in human PDL cells (12). As shown in Fig. 5A, MPDL22 cells without RJ and LPS stimulation expressed low level of CD54 (sample ID. 1), and treatment with only RJ had no effect on CD54 expression (sample ID. 2). MPDL22 cells with LPS strongly enhanced expression of CD54 (sample ID. 3). When we evaluated the expression of CD54 in MPDL22 cells pretreated with RJ 1h prior to LPS stimulation, pretreatment with RJ reversed the LPS-induced upregulation of CD54 (sample ID. 4).

DISCUSSION

In the present study, we observed that RJ increased the formation of mineralized nodules in mouse PDL cells by enhancing osteoblastic differentiation. Additionally, RJ significantly suppressed LPS-induced inflammatory cytokine and chemokine production by MPDL22 cells.

Real-time PCR revealed that RJ enhanced the mRNA expression of osteopontin, osteocalcin, and osterix. Osteopontin is an indicator of bone turnover, and osteocalcin is involved in extracellular matrix mineralization. Osterix is an osteoblast-specific transcription factor that plays an important role in modulating bone formation and osteoblastic differentiation. Upregulation of the above-mentioned mRNAs by RJ correlated with the mineralization of MPDL22 cells. A previous study showed that RJ has estrogenic effects including the prevention of osteoporosis in ovariectomized rats (7, 15). Additionally, RJ has been reported to activate bone metabolism-related genes in the mouse osteoblast-like cell line, MC3T3-E1 (19). Because RJ comprises many components, the effects of RJ on promoting the expression of osteoblastic markers and mineralization in MPDL22 cells may be independent of its estrogenic activity. Isolation and characterization of

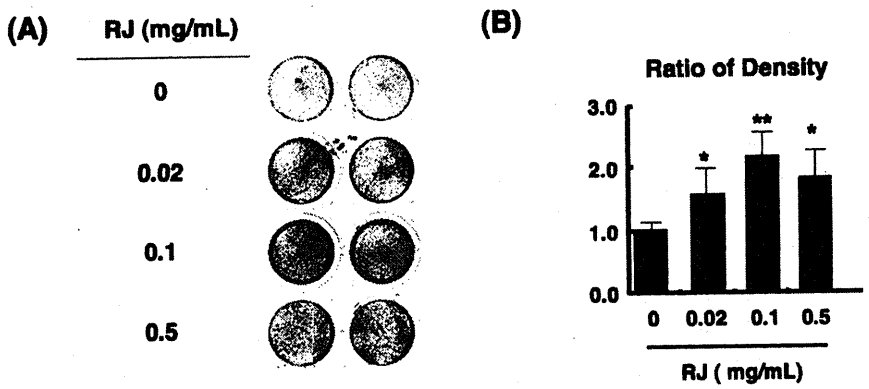

Fig. 3 Effect of RJ on mineralization of MPDL22 cells. (**A**) Effect of RJ (0.02, 0.1, 0.5 mg/mL) on mineralization in MPDL22 cells was examined by alizarin red staining after 12 days of culture in mineralization medium. Results show a representative experiment out of four identical assays. (**B**) The relative intensity of alizarin red staining was determined by image analysis. The data shown were quantified and normalized to those of alizarin red staining without RJ. RJ significantly enhanced mineralized nodule formation in MPDL22 cells. The values are the means ± SD of four different experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with MPDL22 cells cultured with mineralization medium in the absence of RJ.

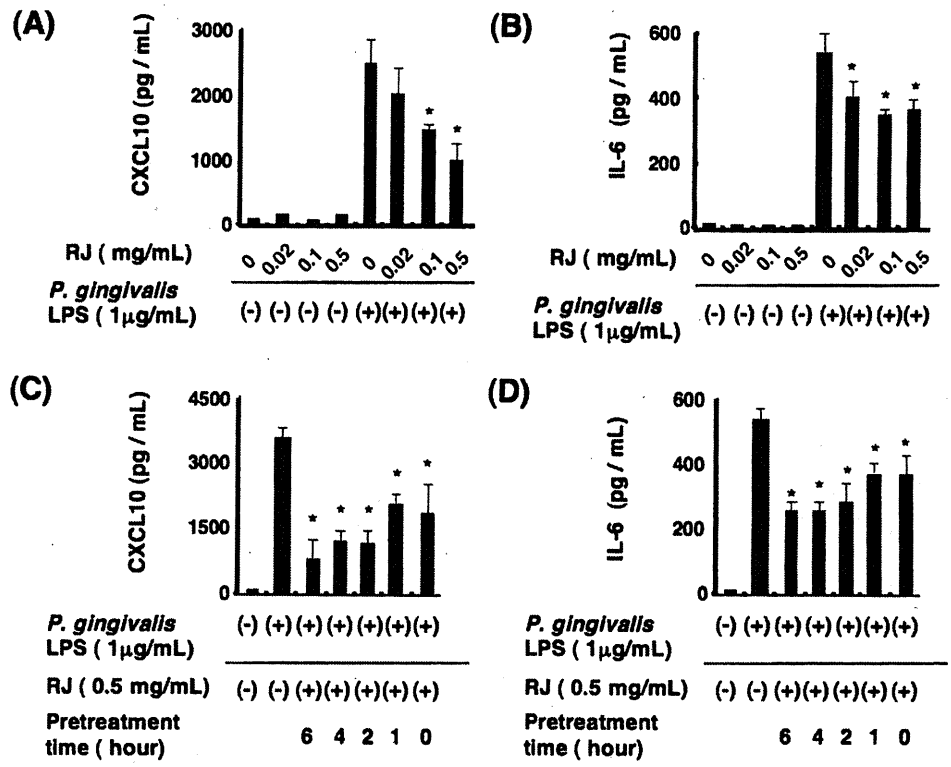

Fig. 4 Effect of RJ on CXCL10 and interleukin-6 (IL-6) production in MPDL22 cells. The effect of RJ on CXCL10 (**A**) and IL-6 (**B**) production in MPDL22 cells. MPDL22 cells were pretreated with RJ prior to LPS stimulation, and supernatants were collected after 24 h. CXCL10 and IL-6 concentrations were determined by ELISA. The results represent the mean values ± SD obtained from triplicate cultures. Next, MPDL22 cells were exposed to 0.5 mg/mL RJ either simultaneously, or up to 6 h before activation with LPS, and supernatants were collected after 24 h. CXCL10 (**C**) and IL-6 (**D**) concentrations were determined by ELISA. The values represent the means ± SD obtained from triplicate cultures. RJ suppressed CXCL10 and IL-6 production in MPDL22 cells. $^*P < 0.05$ compared with LPS-stimulated MPDL22 cells in the absence of RJ.

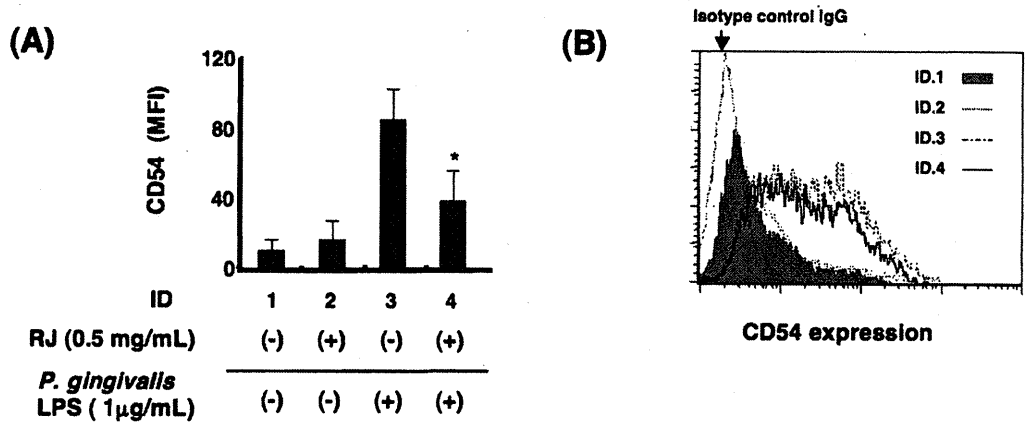

Fig. 5 Effect of RJ on CD54 expression in MPDL22 cells. MPDL22 cells were treated with RJ (0.5 mg/mL) and/or LPS (1 μg/mL). MPDL22 cells were collected after 24 h. The expression levels of CD54 in MPDL22 cells were determined by flow cytometry. (**A**) The results are expressed as mean fluorescent intensity (MFI). The data represent the mean ± SD obtained from three different experiments. $^*P < 0.05$ compared with LPS-stimulated MPDL22 cells in the absence of RJ. (**B**) Fluorescence histogram of CD54 on surface of MPDL22 cells. RJ suppressed CD54 expression in MPDL22 cells stimulated with LPS. The istgramdata is representative of three different experiments. ID. 1: non-treated MPDL22 cells, ID. 2: RJ-treated MPDL22 cells, ID. 3: LPS-stimulated MPDL22 cells, ID. 4: LPS-stimulated MPDL22 cells after RJ-treatment.

the estrogenic compounds in RJ should aid in the understanding of its involvement in bone metabolism.

P. gingivalis is a black-pigmented Gram-negative anaerobe and one of the causative microorganisms for the development of chronic inflammation in the periodontium (14). This is the first report that LPS of *P. gingivalis* can induce secretion of IL-6 and CXCL10 from mouse PDL cells. In human PDL cells, some reports have documented that stimulation by LPS derived from *P. gingivalis* (27) or *Escherichia coli* (23) induced IL-6, IL-8, IL-1β and tumor necrosis factor (TNF)-α production. In this study, we also revealed that RJ possesses inhibitory effects on LPS-stimulated IL-6 and CXCL10 production. IL-6 is a pleiotropic cytokine with a wide range of biological functions including immune responses and bone metabolism (1). CXCL10 is a chemoattractant for T helper1 cells, which are suggested to be involved in alveolar bone destruction in periodontal diseases (9). A previous study reported that RJ suppressed IL-6 and TNF-α production by LPS and interferon-γ stimulated mouse macrophages (11), which is consistent with our observation.

CD54 is a cell surface adhesion molecule whose expression is increased at inflammatory sites such as in the periodontal tissues of periodontitis patients (6). Bacterial components or IL-1β have been reported to upregulate CD54 expression in human PDL cells (8, 12). Consistently, we found that LPS of *P. gingivalis* induced CD54 expression in MPDL22 cells. Furthermore, we revealed that RJ reduced CD54 expression in LPS-stimulated MPDL22 cells (Fig. 5). As anti-inflammatory compounds in RJ, we speculate that adenosine and adenosine derivatives, which have been identified as active components in RJ (5), could be candidates for suppressing CD54 expression in MPDL22 cells. Supporting this, our previous study showed that IL-1β-induced CD54 expression in human gingival fibroblasts was abrogated by adenosine (16). Adenosine and/or other anti-inflammatory compounds in RJ may contribute to the reduction of inflammatory responses in periodontal lesions.

In summary, our present results showed that RJ enhanced osteoblastic metabolism and exerted anti-inflammatory effects. This work suggests that several constituents in RJ may be of benefit to oral and periodontal health. Elucidation of the mechanisms of the effects of RJ in mouse and human PDL cells requires further investigation.

Acknowledgments

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Nicotine up-regulates IL-8 expression in human gingival epithelial cells following stimulation with IL-1β or *P. gingivalis* lipopolysaccharide via nicotinic acetylcholine receptor signalling

Yoichiro Kashiwagi, Manabu Yanagita, Yuko Kojima, Yoshio Shimabukuro, Shinya Murakami*

Department of Periodontology, Division of Oral Biology and Disease Control, Osaka University Graduate School of Dentistry, 1-8 Yamadaoka, Suita, Osaka 565-0871, Japan

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ABSTRACT

Objective: Cigarette smoking is an important risk factor for periodontal disease. The aim of this study is to evaluate the effect of nicotine, a major component of cigarette smoke, on interleukin-8 (IL-8) production and cellular signalling via nicotinic acetylcholine receptors (nAChRs) in human gingival epithelial cells (HGECs).

Design: Messenger RNA (mRNA) expression of nAChR subunits in three different HGEC lines (epi 4, Tfx and E6E7) was assessed using reverse transcription-polymerase chain reaction (RT-PCR). HGECs were stimulated by 1×10^{-3} M nicotine in the presence or absence of IL-1β or *Porphyromonas gingivalis* lipopolysaccharide (LPS). IL-8 production was then examined using real-time PCR and enzyme-linked immunosorbent assay. Nicotine-mediated signalling in the epi 4 cell line was also evaluated by Western blotting.

Results: HGECs expressed several nAChR subunits. Nicotine increased the secretion of IL-8 from HGECs that were cultured in the presence of IL-1β or *P. gingivalis* LPS and also induced the phosphorylation of extracellular signal-regulated kinase (ERK) in epi 4. Pretreatment with non-selective nAChR antagonist or intracellular calcium chelator reduced the nicotine-induced phosphorylation of ERK. Furthermore, nicotine-induced IL-8 secretion was decreased by pretreatment with non-selective nAChR antagonist, ERK1/2 inhibitor or intracellular calcium chelator.

Conclusion: These findings indicate that nicotine increases IL-8 production in gingival epithelial cells via ERK phosphorylation following Ca^{2+} signalling after nAChR activation.

1. Introduction

Gingival epithelial cells play an important role in homeostasis of periodontal tissue. They prevent entry of periodontopathogen into periodontal tissue by forming a physical barrier and provide nonspecific, rapid host defence reaction resulting in recruitment of professional immune cells such as macrophages, dendritic cells and lymphocytes.[1–3] Several lines of evidence have indicated that bacterial stimulation induces pro-inflammatory cytokines (interleukin (IL)-1β, IL-6, IL-8 and tumour necrosis factor-α) in oral and gingival epithelial cells.[4,5] In addition, we have previously revealed that human gingival epithelial cells (HGECs) and oral epithelial cell line (KB

* *Corresponding author.* Tel.: +81 6 6879 2932; fax: +81 6 6879 2934.
E-mail address: yanagita@dent.osaka-u.ac.jp (M. Yanagita).
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cells) produced IL-6, IL-8 and monocyte chemoattractant protein-1 after stimulation with *Porphyromonas gingivalis*, a causative pathogenic microorganism associated with periodontal disease.[6,7] Amongst the cytokines and chemokines produced by epithelial cells, IL-8, a neutrophil chemoattractant and activator, plays the crucial role in the first line of host defence against microorganisms.[8] Furthermore, a report demonstrating that constitutive IL-8 production was detected in non-inflamed gingival epithelium has suggested that HGECs have the ability of immunosurveillance in periodontal tissues.[9]

Cigarette smoking is an important environmental risk factor in the development of periodontal diseases.[10] Cigarette smoke consists of thousands of chemicals which can affect periodontal tissue. Amongst these, nicotine is the main constituent of cigarette smoke and a selective agonist of nicotinic acetylcholine receptors (nAChRs). Interestingly, nicotine has been documented to have an immunomodulating function, suppressing macrophage Th1-type immune responses towards Th2.[11] Although gingival epithelium is the first tissue exposed to nicotine following inhalation of cigarette smoke, little is known about the effect of nicotine on the cellular function of gingival epithelial cells, especially in terms of the innate immune response.

Chemical insults such as nicotine exposure during smoking in the presence of a bacterial plaque may affect the gingival tissue by altering the innate immune system of HGECs and may facilitate progression of periodontal diseases. In this study, we examined the effects of nicotine exposure on IL-8 production as the gingival epithelial innate immune response following stimulation with the pro-inflammatory cytokine (IL-1β) and bacterial components (*P. gingivalis* lipopolysaccharide (LPS)).

2. Materials and methods

2.1. Cell culture

All human subjects who participated in this study provided informed consent for the protocol reviewed and approved by the Institutional Review Board of the Osaka University Graduate School of Dentistry. Gingival tissue specimens were obtained from three different patients with chronic periodontitis (one male and two females; average age = 45 years) at distal wedge operation for therapeutic purposes. All patients were systemically healthy and non-smokers. Two or three gingival tissue specimens per patient were minced and treated with 0.4% dispase II (Boehringer Mannheim GmbH, Mannheim, Germany) overnight at 4 °C. The epidermal sheet was separated and trypsinised with 0.05% Trypsin-ethylene diamine tetraacetic acid (Trypsin-EDTA) (Life Technologies, Rockville, MD, USA) so that single cells would be dispersed. The cells were then seeded and subcultured in a 25-cm^2 flask (Corning Inc., Corning, NY, USA). The HGECs were grown in keratinocyte-specific growth media (HuMedia KG2, Kurabo, Osaka, Japan) containing final concentrations of 0.5 $\mu g\,ml^{-1}$ hydrocortisone, 10 $\mu g\,ml^{-1}$ insulin, 0.4% (v/v) bovine pituitary extract, 0.1 $ng\,ml^{-1}$ human epidermal growth factor (hEGF), 50 $\mu g\,ml^{-1}$ gentamycin and 50 $ng\,ml^{-1}$ 1 amphotericin B. The HGEC cell line, epi4, has previously been established.[6,12] The remaining two HGEC cultures were transformed by the SV40 T antigen using Tfx™-20 (Promega Corporation, Madison, WI, USA), and transfected with human papillomavirus 16 (HPV-16) E6 and E7 open reading frames. Transfection was performed using a retroviral system for HPV-16, named Tfx and E6E7, respectively which was kindly provided by Dr. M. Saito (Tokyo University of Science, Tokyo, Japan).[13] These cell lines survived for more than 150 culture passages. No changes in cellular characteristics were detected after culture passages.

2.2. Cell stimulation

HGEC cell lines were seeded in culture plates at a similar density for each experiment and were then grown to subconfluence. The cultured HGECs were then grown in keratinocyte-specific growth media in the absence of growth factors for 12 h. For the detection of messenger RNA (mRNA) expression of IL-8 and measurement of IL-8 production, three HGEC cell lines (epi4, Tfx, and E6E7) were treated with 0.1 $ng\,ml^{-1}$ human recombinant IL-1β (R&D System, Inc., Minneapolis, MN, USA), or 10 $\mu g\,ml^{-1}$ *P. gingivalis* LPS (Invitrogen, San Diego, CA, USA) in the presence or absence of nicotine (1×10^{-6} M, 1×10^{-3} M: Sigma–Aldrich Inc., St Louis, MO, USA). Total RNA was isolated from each well after incubation for 12 h, and the culture supernatants were harvested after incubation for 24 h. In some experiments, epi4 cells were pretreated for 1 h in the presence of a non-selective nAChR antagonist: d-tubocurarine (Sigma–Aldrich Inc.), an intracellular calcium chelator: BAPTA-AM (Dojindo, Kumamoto, Japan) or an extracellular signal-regulated kinase1/2 (ERK1/2) inhibitor: U0126 (Promega Corporation), prior to stimulation with IL-1β and nicotine. The optimal time points and the concentrations of IL-1β and *P. gingivalis* LPS were determined based on preliminary experiments for detection of IL-8 expression and IL-8 secretion.

2.3. Reverse transcription-polymerase chain reaction analysis

The total RNA of three HGEC lines was isolated from cultured cells using a prepared phenol–chloroform solution (RNABee: Tel-Test, Inc., Friendship, TX, USA), according to the manufacturer's instructions. The precipitated RNA was resolved in 0.1% diethylpyrocarbonate-treated distilled water. Complementary DNA (cDNA) synthesis and amplification via polymerase chain reaction (PCR) were performed according to previously described methods.[14,15] Primer sequences were described previously by Yanagita et al.[15] Human brain RNA (Biochain Institute Inc., Hayward, CA, USA) was used as positive control.

2.4. Real-time PCR analysis

Isolation of total RNA and cDNA synthesis were performed using the methods described above. PCR reactions were carried out using the ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Green PCR Master

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Mix (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. All reactions were run in triplicate. Specific primers for human IL-8 (forward primer: 5′-ACACTGCGCCAACACAGAAATTA-3′, reverse primer: 5′-TTTGCTTGAAGTTTCACTGGCATC-3′) and hypoxanthine–guanine phosphoribosyl transferase (HPRT) (forward primer: 5′-GGCAGTATAATCCAAAGATGGTCAA-3′, reverse primer: 5′-GTCAAGGGCATATCCTACAACAAAC-3′) were purchased as pre-designed products (Takara Bio Inc., Shiga, Japan). HPRT served as a housekeeping gene.

2.5. *Measurement of IL-8 secretion*

Cytokine levels were determined by using Human IL-8 Elipair™ (R&D Systems) by following the manufacturer's protocol. All reactions were run in triplicate.

2.6. *Investigation of mitogen-activated protein kinase (MAPK) phosphorylation*

Epi 4 cells were seeded on a six-well plate and grown to confluence in keratinocyte-specific growth media supplemented with specific growth regents. After 12 h, cells were incubated in growth media without the supplemental reagents for a further 12 h. Cells were then stimulated with or without 1×10^{-3} M nicotine for 5, 10, 15, 30 and 60 min. Cells were rinsed with ice-cold PBS and lysed with radio immunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 5 mM EDTA, 1% NP-40, 0.5% sodium dodecyl sulphate (SDS), 1% deoxycholate) containing protease inhibitors (10 $\mu g\ ml^{-1}$) phenylmethylsulphonylfluoride (PMSF), 30 $\mu g\ ml^{-1}$ aprotinin, a phosphatase inhibitor and 1 mM sodium orthovanadate (Sigma–Aldrich Inc.). Protein was quantified using the Bradford assay. To determine phosphorylation of 21 mitogen-activated protein kinases (MAPKs), we used the Human Phospho-MAPK Array Kit (R&D Systems), according to the manufacturer's protocol. We detected immunoreactive proteins using a Western blotting detection system (ECL Plus, Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.7. *Western blotting for ERK*

Epi 4 cells were stimulated with or without 1×10^{-3} M nicotine for 5, 10, 15, 30 and 60 min. In some experiments, the inhibitor, a non-selective nAChR antagonist or an intracellular calcium chelator, was added to the cultures 1 h before stimulation with nicotine. Cells were rinsed with ice-cold phosphate buffered saline (PBS) and lysed with RIPA buffer. Equal amounts of protein (40 μg per lane) were separated using sodium dodecyl sulphate-polyacryalamide gel electrophoresis (SDS-PAGE), then electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). After blocking with PBS-Tween (PBS-T) buffer containing 5% non-fat milk, membranes were incubated with primary rabbit anti-ERK1/2 antibody (Cell Signaling Technology Inc., Danvers, MA, USA) and rabbit anti-phospho ERK1/2 antibody (Cell Signaling Technology Inc., Danvers, MA, USA) overnight at 4 °C. Membranes were then washed briefly and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG) antibody (GE Healthcare JAPAN, Tokyo, Japan). We detected immunoreactive proteins using a Western blotting detection system, and densitometrically analysed bands with image analysis software (Quantity One, Bio-rad, Hercules, CA, USA).

2.8. *Statistical analyses*

Statistical analyses were performed using Dunnett's test for comparison. Differences with a p value <0.05 were considered significant.

3. Results

3.1. *Expressions of nAChR mRNA in three HGEC lines*

Three gingival epithelial cell lines were examined for the expressions of nAChR subunit mRNA using reverse transcription polymerase chain reaction (RT-PCR). Brain mRNA was

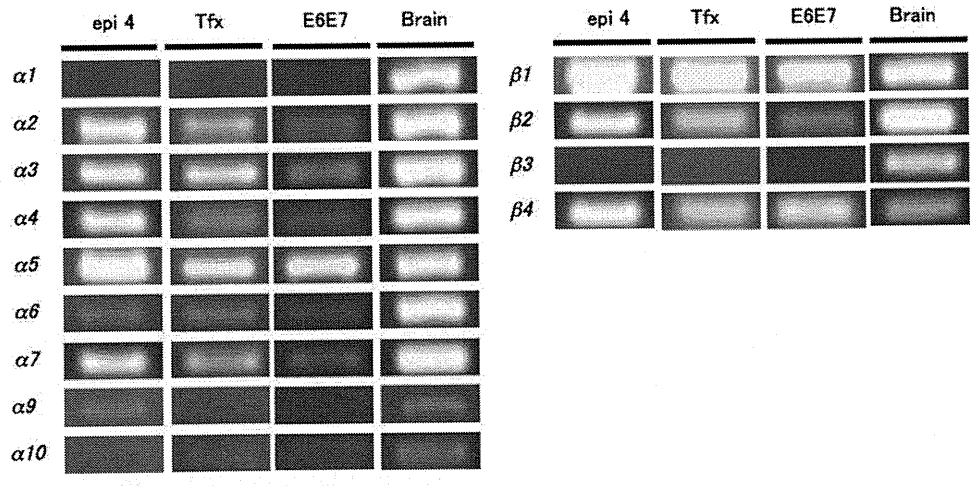

Fig. 1 – Expression profiles of nAChR mRNA in three different HGEC lines. Total RNA was extracted from subconfluent cultured cells. The expression of nAChR subunit mRNA was analysed by RT-PCR using specific primer sets. Brain RNA was used as a positive control.

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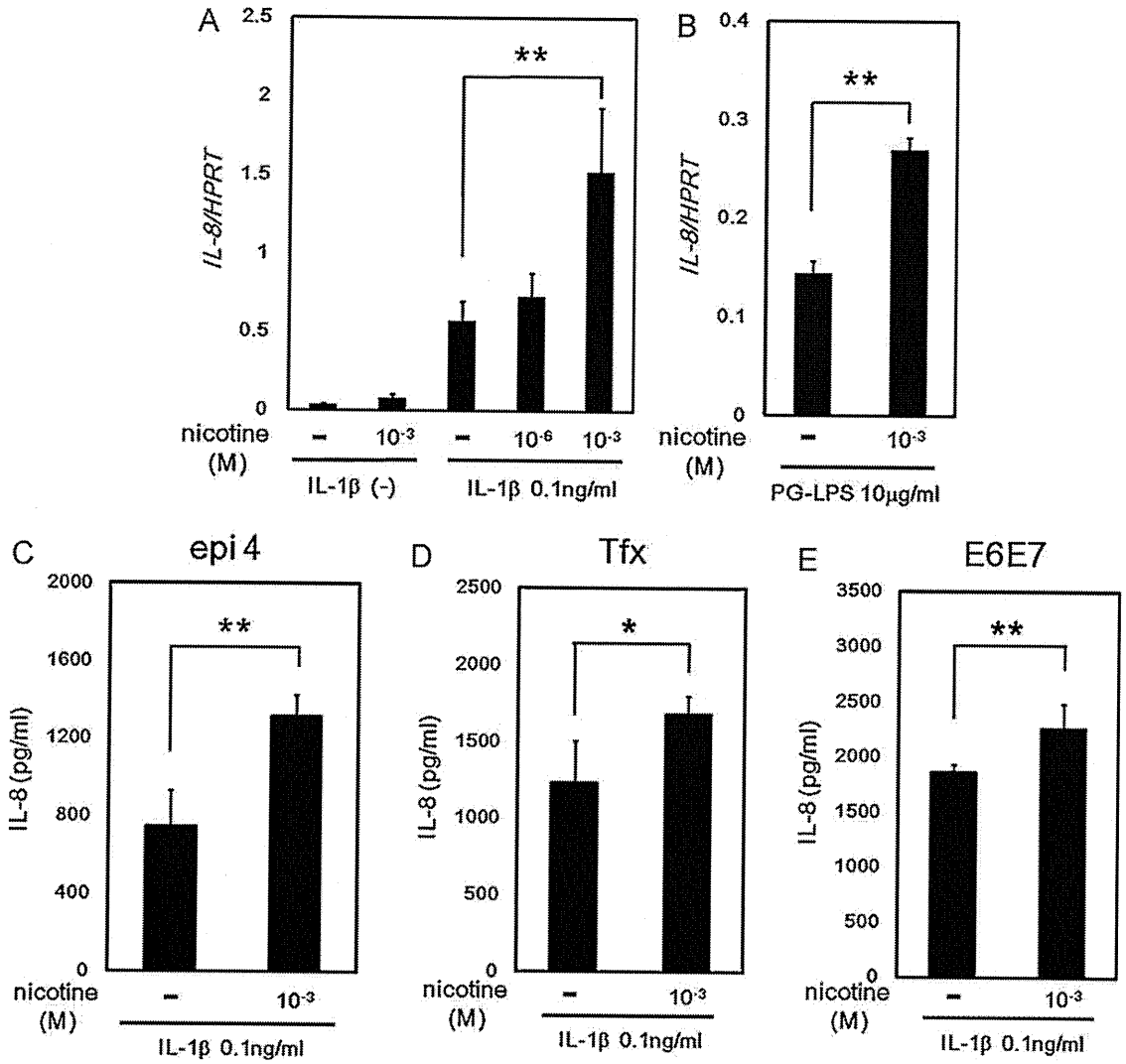

Fig. 2 – Nicotine up-regulates the expression of IL-8. IL-8 mRNA expression in epi 4 stimulated with IL-1β (A) or *P. gingivalis* LPS (B: PG-LPS) in the presence or absence of nicotine for 12 h was quantified using real-time PCR. Data are expressed as mean ± SD of three determinations. **$p < 0.01$ compared with IL-1β or *P. gingivalis* LPS alone. IL-8 production in supernatants of epi 4 (C), Tfx (D), and E6E7 (E) stimulated with IL-1β in the presence or absence of nicotine for 24 h. Concentrations of IL-8 were measured using ELISA. Data are expressed as mean ± SD of three determinations. *$p < 0.05$, **$p < 0.01$ compared with IL-1β or *P. gingivalis* LPS alone.

used as a positive control for nAChRs. Fig. 1 shows representative PCR products from three immortalised HGEC lines, epi 4, Tfx and E6E7 cells. We found that all three HGEC lines expressed mRNA for several different nAChR subunits; α2-7, α9, β1, β2 and β4 subunit mRNA were found in epi 4, whilst α2-7, α10, β1, β2 and β4 subunit mRNA were found in Tfx and E6E7.

3.2. *Effects of nicotine on IL-8 expression in HGEC lines*

We initially confirmed that nicotine (10^{-8}–10^{-3} M at 24 h) did not affect the viability of HGEC lines in our preliminary experiments. To examine the effects of nicotine on the expression of IL-8 mRNA, we extracted total RNA and performed real-time PCR for epi 4, Tfx and E6E7 cells. As shown in Fig. 2(A), 1×10^{-3} M nicotine slightly induced an increase in IL-8 mRNA expression as compared with nicotine-free conditions. Interestingly, however, in epi 4 cells that had been cultured with 0.1 ng ml^{-1} IL-1β and nicotine (1×10^{-6} M and 1×10^{-3} M), IL-8 mRNA expression was significantly enhanced as compared with IL-1β alone (Fig. 2(A)). We then examined the effect of nicotine on epi4 stimulated with *P. gingivalis* LPS. As shown in Fig. 2(B), IL-8 mRNA expression in epi 4 cells was also significantly elevated after 12 h of culture in the presence of nicotine and *P. gingivalis* LPS as compared with *P. gingivalis* LPS alone. As shown in Fig. 2(C), in the presence of IL-1β, nicotine up-regulated the secretion of IL-8 from epi 4 cells. The enhancement in IL-8 production by nicotine was also detected in Tfx, and E6E7 cells (Fig. 2(D) and (E)). Since all three HGEC lines were shown to have similar expression of nAChR subunits and IL-8 production, epi 4 cells were used for all subsequent experiments.

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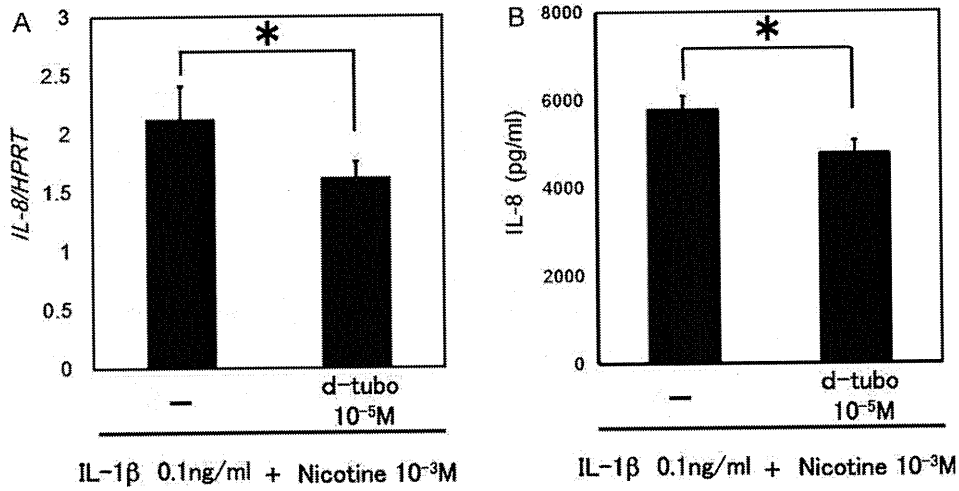

Fig. 3 – d-Tubocurarine, a nonselective nAChR antagonist, inhibits nicotine-induced upregulation of IL-8. After pretreatment with d-tubocurarine for 1 h, epi 4 was stimulated with IL-1β and nicotine for 12 h (A: real-time PCR) or 24 h (B: ELISA). Data are expressed as mean ± SD of three determinations. *$p < 0.05$ compared with IL-1β and nicotine alone.

3.3. *nAChRs are involved in IL-8 mRNA and protein secretion induced by nicotine in epi 4 cells*

We further investigated whether these effects of nicotine were mediated through nAChRs. We found that the up-regulation of IL-8 mRNA expression and protein secretion from epi 4 cells, which depended on stimulation with nicotine and IL-1β, were reduced by a non-selective nAChR antagonist (Fig. 3(A) and (B)). These results indicated that nicotine enhanced the inflammatory effect of IL-1β on epi 4 through nAChRs.

3.4. *Cell signalling molecules, related to activation by nicotine, in epi4 cells*

MAPK phosphorylation in nicotine-stimulated HGECs was examined to evaluate the functional significance of nAChR in HGECs. A strong ERK1/2 signal was detected using a Human Phospho-MAPK Array Kit™, as shown in Fig. 4. To examine whether the activation of ERK was involved in nicotine-induced IL-8 release, protein extracts prepared from epi 4 cells, which had been incubated with 1×10^{-3} M nicotine, were immunoblotted with antibodies against p-ERK1/2 and total ERK1/2. p-ERK1/2 levels increased after 10 min of incubation with nicotine (Fig. 5). This nicotine-induced ERK phosphorylation was suppressed in the presence of either d-tubocurarine or BAPTA-AM (Fig. 5). These results confirmed that nicotine-induced activation of ERK was associated with Ca^{2+} signalling via nAChRs. Interestingly, as shown in Fig. 6, an increase in nicotine-induced IL-8 production from epi 4 cells was significantly reduced in the presence of either an intracellular calcium chelator or a selective inhibitor of MAPK/ERK kinase (MEK) as compared with that of IL-1β alone.

4. Discussion

Recent works have shown that keratinocytes or epithelial cells express nAChR. Several studies reported that α1, α3-7, α9 and

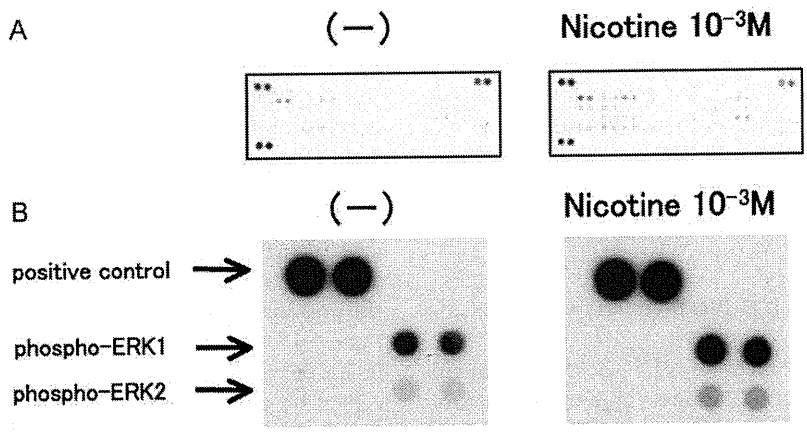

Fig. 4 – Investigation of MAPK phosphorylation in nicotine-stimulated epi4 cells. epi 4 cells were cultured in the presence or absence of nicotine for 10 min. To investigate phosphorylation of 21 MAPKs, a Human Phospho-MAPK Array Kit™ was utilized. All data (A), the enlargement of the area blotted for p-ERK1/2 and positive control (B) are shown.

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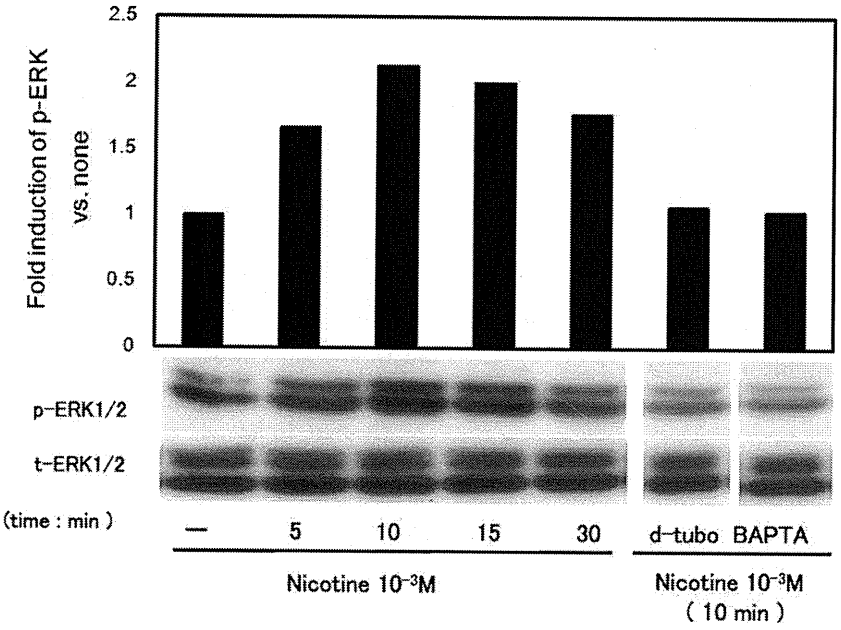

Fig. 5 – d-Tubocurarine and BAPTA-AM inhibit nicotine-induced upregulation of ERK1/2 phosphorylation. After pretreatment with d-tubocurarine or BAPTA-AM for 1 h, epi 4 cells were stimulated with nicotine for 10 min. Cell lysate was subjected to immunoblotting for p-ERK. Blotting images were analysed densitometrically.

10 and β1, 2 and 4 nAChR subunits were present in human airway epithelial cells.[16–18] Nguyen et al.[19] characterised nAChR on gingival keratinocytes and oesophageal epithelia and found the expression of α3, α5, α7 and β2 nAChR subunits. In this study, we established three HGEC lines from three different patients to detect nAChR expression and IL-8 production in these cells. These studies confirmed that these three HGECs showed the same nAChR expression and IL-8 production phenotype. RT-PCR experiments revealed that α2-7, β1, 2 and 4 subunit mRNAs are expressed in three established HGEC lines. Two additional subunits, α9 and α10, were also expressed in epi 4, and in Tfx and E6E7, respectively. The subtle difference in nAChR subunit expression may be caused by the difference in differentiation stage of the HGECs examined. To our knowledge, this is the most extensive analysis of nAChR subunit expression in HGECs. Furthermore, we demonstrated that the non-selective nAChR antagonist, d-tubocurarine, suppressed nicotine-induced IL-8 production and enhanced phosphorylation of ERK. This suggests that the effect of nicotine on HGECs can be transmitted through nAChRs on the cell surface.

It is well known that gingival or oral epithelial cells can secrete IL-8 in response to several periodontal pathogens or pro-inflammatory cytokines.[20,21] Our previous study reported that *P. gingivalis* LPS can induce IL-8 production in HGECs via Toll-like receptor-2.[6] To explore the effect of nicotine on pro-inflammatory cytokine production in HGECs, we used IL-1β and *P. gingivalis* LPS as stimulants to induce the synthesis of IL-8 in this study. Consistent with the previous studies, our results showed that both IL-1β and *P. gingivalis* LPS can up-regulate IL-8 production in three established HGECs. In addition, IL-8 expression was enhanced in the presence of nicotine in a dose-dependent manner. Nicotine has been shown to attenuate IL-8 production following LPS stimulation in activated monocytic cells.[22] Furthermore, nicotine inhibited the production of pro-inflammatory cytokines via nicotine signalling.[23] Conversely, nicotine has been reported to stimulate neutrophils and gingival fibroblasts to produce IL-8.[24,25] In addition, Mahanonda et al.[26] reported that nicotine and cigarette smoke extract stimulated IL-8 expression in HGEC cultures, which is consistent with this report. This discrepancy in the effect of nicotine may be dependent on cell type and the difference in expression levels of nuclear factor (NF)-κB, which controls inflammatory cytokine gene transcription.[27] Nicotine prevented activation of the NF-κB pathway in professional antigen-presenting cells, such as macrophages,[23,24] whereas it stimulated NF-κB activation in neutrophils, the innate immune sentinels. Like neutrophils, HGECs, which are the primary interface between gingival tissue and the oral cavity, can sense pathogens and chemical insults. Because these cells play an important role in providing the first line of host defence, they may sense nicotine as a foreign stress and induce a pro-inflammatory response to maintain homeostasis. Further studies are required to elucidate the mechanism responsible for the diversity in effects of nicotine amongst different cell types.

Nicotine has been reported to activate ERK1/2 in oral keratinocytes.[28] In addition, Ca^{2+} influx can be induced after the binding of nicotine to nAChR.[29] Amongst previous studies of signal transduction via nAChR subunits, the α7 nAChR subunit, which can form homopentameric α7 nAChR, has been well documented. For example, the interaction with α7 nAChR stimulates JAK-2-signal transducer and activator of transcription 3-suppressor of cytokine signalling 3 (JAK-2-STAT-3-SOCS3) pathway in macrophage.[23,30] In oral keratinocytes, α7 nAChR can use Ras/Raf-1/MEK1/ERK and JAK-2/STAT-3 signalling pathways.[28] α7 nAChR has also been reported to activate phosphatidylinositol-3 kinase (PI3K), a

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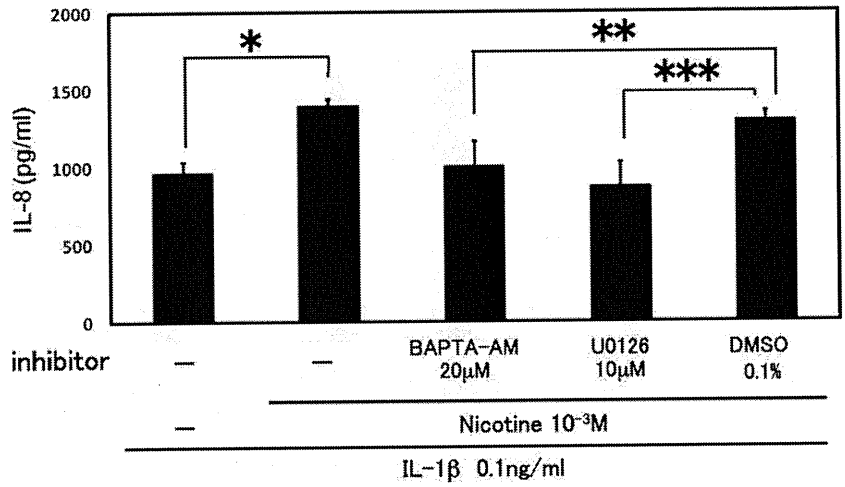

Fig. 6 – BAPTA-AM and U0126 inhibit nicotine-induced up-regulation of IL-8 production. After pretreatment with BAPTA-AM and U0126 for 1 h, epi 4 cells were stimulated with IL-1β in the presence or absence of nicotine for 24 h. The concentration of IL-8 was evaluated using ELISA. Data are expressed as mean ± SD of three determinations. *$p < 0.05$ compared with IL-1β alone, **$p < 0.01$ and ***$p < 0.05$ compared with DMSO.

Ca^{2+}-dependent kinase in neuronal cells.[31] In the present study, nicotine rapidly induced the activation of ERK1/2 phosphorylation in the HGEC line, epi 4. Furthermore, nicotine-induced phosphorylation in epi 4 was suppressed by pretreatment with a non-selective nAChR antagonist or an intracellular calcium chelator. Previous study has documented that nicotine induces an elevation in Ca^{2+} levels via nAChRs, which is dependent on the activation of a voltage-operated Ca^{2+} channel, and also involves Ca^{2+} release from intracellular stores.[32] Our study showed that both signalling via nAChR and Ca^{2+} release from intracellular stores were at least involved in nicotine-induced IL-8 production and ERK1/2 phosphorylation in epi4 cells. This result suggests that nicotine-induced IL-8 production and ERK1/2 activation in HGECs is dependent on Ca^{2+} signalling, possibly via nAChRs.

The present findings demonstrate that HGECs express an array of nAChR subunits that can temporarily transmit nicotine signalling to synergistically induce the secretion of IL-8 in the presence of IL-1β or P. *gingivalis* LPS. However, further studies regarding the effect of smoking, and therefore nicotine on cellular characteristics in HGECs, are required. In particular, the effect of the long-term exposure of nicotine, or the other cigarette smoke constituents (e.g., carbon monoxide, acetaldehyde, acrolein and so on) on HGECs needs to be investigated. These further studies may clarify the mechanism for initiation and progression of periodontal diseases.

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Competing interests

None declared.

Ethical approval

Not required.

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Nicotine modulates the immunological function of dendritic cells through peroxisome proliferator-activated receptor-γ upregulation

Manabu Yanagita, Ryohei Kobayashi, Yuko Kojima, Kenta Mori, Shinya Murakami *

Department of Periodontology, Division of Oral Biology and Disease Control, Osaka University Graduate School of Dentistry, Yamadaoka 1-8, Suita, Osaka 565-0871, Japan

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ABSTRACT

We examined the effects of nicotine on differentiation and function of monocyte-derived human dendritic cells (DCs). NiDCs, which were the DCs differentiated in the presence of nicotine, showed lower levels of CD1a. Secretion of IL-12 and TNF-α by lipopolysaccharide (LPS)-stimulated NiDCs was significantly suppressed compared to monocyte-derived DCs grown without nicotine. NiDCs displayed a diminished capacity to induce allogeneic T cell proliferation with a reduced production of IFN-γ, and maintained/enhanced LPS-mediated expression of coinhibitory molecules. Interestingly, NiDCs enhanced the expression of nuclear receptor peroxisome proliferator-activated receptors γ (PPAR γ), which has immunomodulatory properties. Expression of PPAR γ and PPAR γ-target genes was significantly inhibited by pretreatment with d-tubocurarine, antagonist of non-selective nicotinic acetylcholine receptors (nAChR). In addition, reduction of Th1 responses was inhibited after blocking nAChR-mediated signal. These data suggest the effect of nicotine on altering DC immunogenicity by impeding Th1 immunity is partially mediated by upregulation of PPAR γ.

1. Introduction

Cigarette smoking significantly increases the risk of developing numerous diseases such as cancer, vascular disease, periodontal disease, and chronic obstructive pulmonary disease (COPD) [1–5]. It has been suggested that the increased incidence of these diseases in smokers may be due to chronic inhalation of chemicals in cigarette smoke that eventually leads to altered immune responses [6]. Among thousands of chemical components in cigarette smoke, nicotine is a main component and is known to induce T-cell anergy and immunosuppression [7]. Nicotine is a selective agonist of the nicotinic acetylcholine receptors (nAChRs). Human nAChRs are pentamers that are also agonist-regulated ion channels. nAChRs are expressed by neuronal as well non-neuronal cells, including epithelial cells [8], lymphocytes [9], alveolar macrophages [10,11], and eosinophils [12]. Recent reports suggest that nicotine inhibits systemic inflammation via an anti-inflammatory, cholinergic pathway coupled to α7nAChRs [11,13]. During inhalation of cigarette smoke, the epithelial surface of the oral cavity, bronchi and lungs are exposed to localized, high doses of nicotine ($>10^{-3}$ M). In particular, nicotine concentrations in the saliva of long-term snuff users can reach mM levels [14–16].

Dendritic cells (DCs) are the most efficient antigen-presenting cells for coupling the innate to the adaptive immune responses [17]. In the presence of bacterial components such as LPS, DC maturation can be induced by stimulation of TLRs expressed on DCs. Matured DCs produce proinflammatory cytokines and up-regulate the expression of costimulatory molecules [18]. In addition, they detect, capture and process foreign antigens and evoke a variety of immunological responses by presenting foreign antigens to naive CD4 T cells, resulting in differentiation into Th1, Th2, regulatory T cells (Treg) and Th17 cells [19]. It is well established that DCs can display unique functional characteristics depending on the different tissue microenvironments to which they are exposed *in vivo* and on different tissue culture conditions *in vitro* [20–22]. Recent studies indicate that cigarette smoke and nicotine suppressed DC-mediated immune responses in human *in vitro* [23,24]. In contrast, another study showed that nicotine strongly activated DC-mediated adaptive immune responses [25]. The difference in effects may be due to the concentration of nicotine used in the experiments. The effects of nicotine on *in vitro*-differentiated DC function remains controversial, however, since those reports suggest the possibility that nicotine modulates DC function regardless of actual nicotine-induced DC activation.

One of the nuclear transcription factors that influence the DC immune function is the peroxisome proliferator-activated receptor-γ (PPAR γ). PPAR γ was originally identified as a promoter of adipose differentiation and regulator of insulin and glucose metabolism [26,27]. Recently, PPAR γ has also been shown to mediate anti-inflammatory effects via negative interference with pro-inflammatory signaling via NF-κB [28,29]. A disruption of the PPAR

* Corresponding author. Fax: +81 6 6879 2934.
E-mail address: ipshinya@dent.osaka-u.ac.jp (S. Murakami).

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