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

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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

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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).

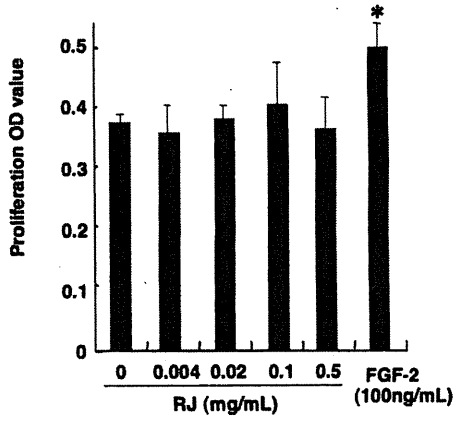

Fig. 1 Effect of RJ on proliferation of MPDL22 cells. MPDL22 cells were cultured with various doses of RJ or FGF-2 (100 ng/mL) for two days. RJ did not induce significant proliferative responses in MPDL22 cells. Values are the means ± SD of four assays. $^*P < 0.05$ compared with medium only.

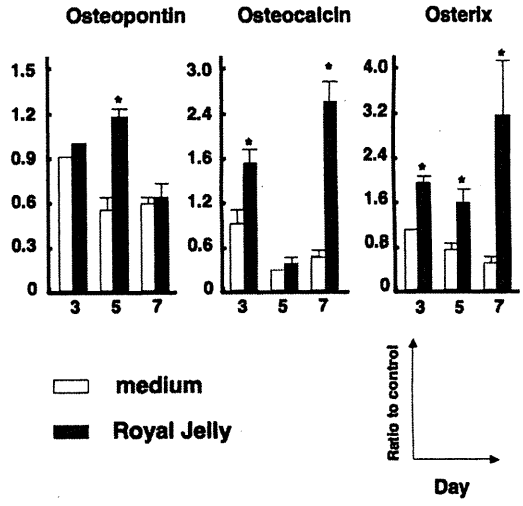

Fig. 2 Analysis of mRNA expression of osteopontin, osteocalcin and osterix in MPDL22 cells. RNA samples were obtained from MPDL22 cells at 3, 5 and 7 days after RJ treatment (0.5 mg/mL). The relative expression of each gene was normalized to the expression levels of HPRT, and the expression of osteopontin, 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

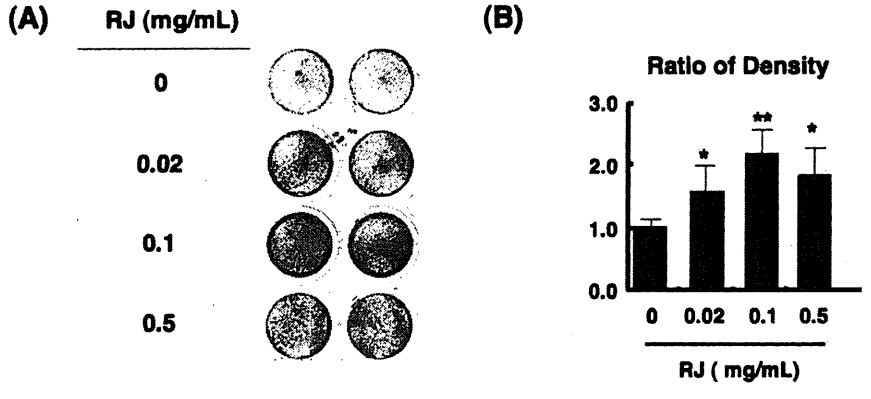

Fig. 3 Effect of RJ on mineralization of MPDL22 cells. (**A**) Effect of RJ (0.02, 0.1, 0.5 mg/mL) on mineralization in MPDL22 cells was examined by alizarin red staining after 12 days of culture in mineralization medium. Results show a representative experiment out of four identical assays. (**B**) 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.

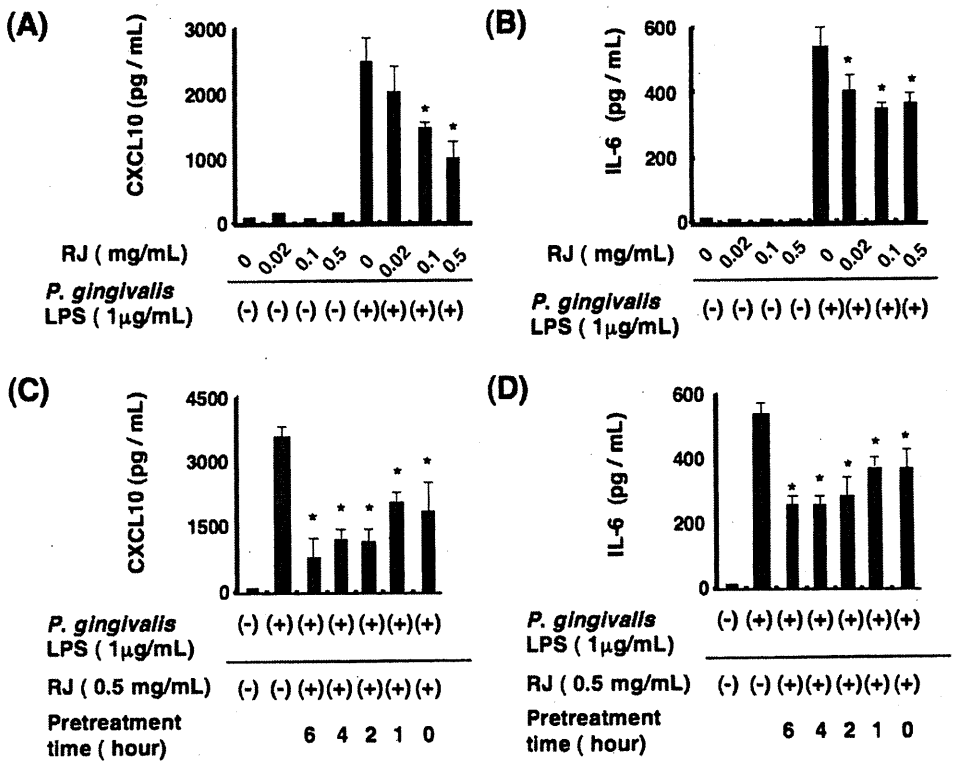

Fig. 4 Effect of RJ on CXCL10 and interleukin-6 (IL-6) production in MPDL22 cells. The effect of RJ on CXCL10 (**A**) and IL-6 (**B**) production in MPDL22 cells. MPDL22 cells were pretreated with RJ prior to LPS stimulation, and supernatants were collected after 24 h. CXCL10 and IL-6 concentrations 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.

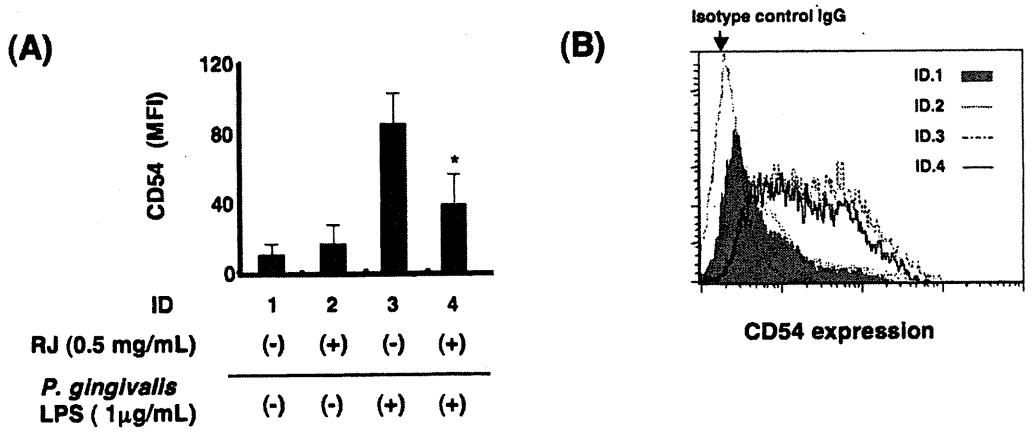

Fig. 5 Effect of RJ on CD54 expression in MPDL22 cells. MPDL22 cells were treated with RJ (0.5 mg/mL) and/or LPS (1 μg/mL). MPDL22 cells were collected after 24 h. The expression levels of CD54 in MPDL22 cells were determined by flow cytometry. (**A**) The results are expressed as mean fluorescent intensity (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

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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

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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

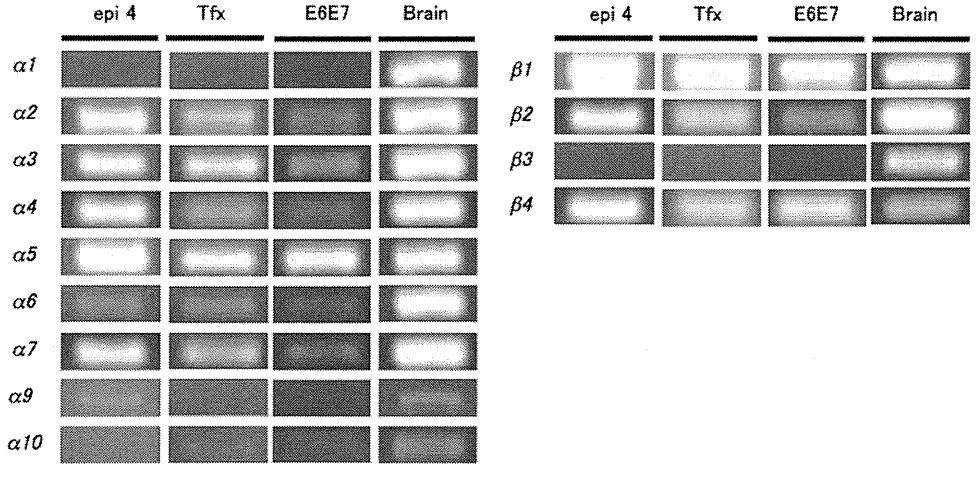

Fig. 1 – Expression profiles of nAChR mRNA in three different HGEC lines. Total RNA was extracted from subconfluent cultured cells. The expression of nAChR subunit mRNA was analysed by RT-PCR using specific primer sets. Brain RNA was used as a positive control.

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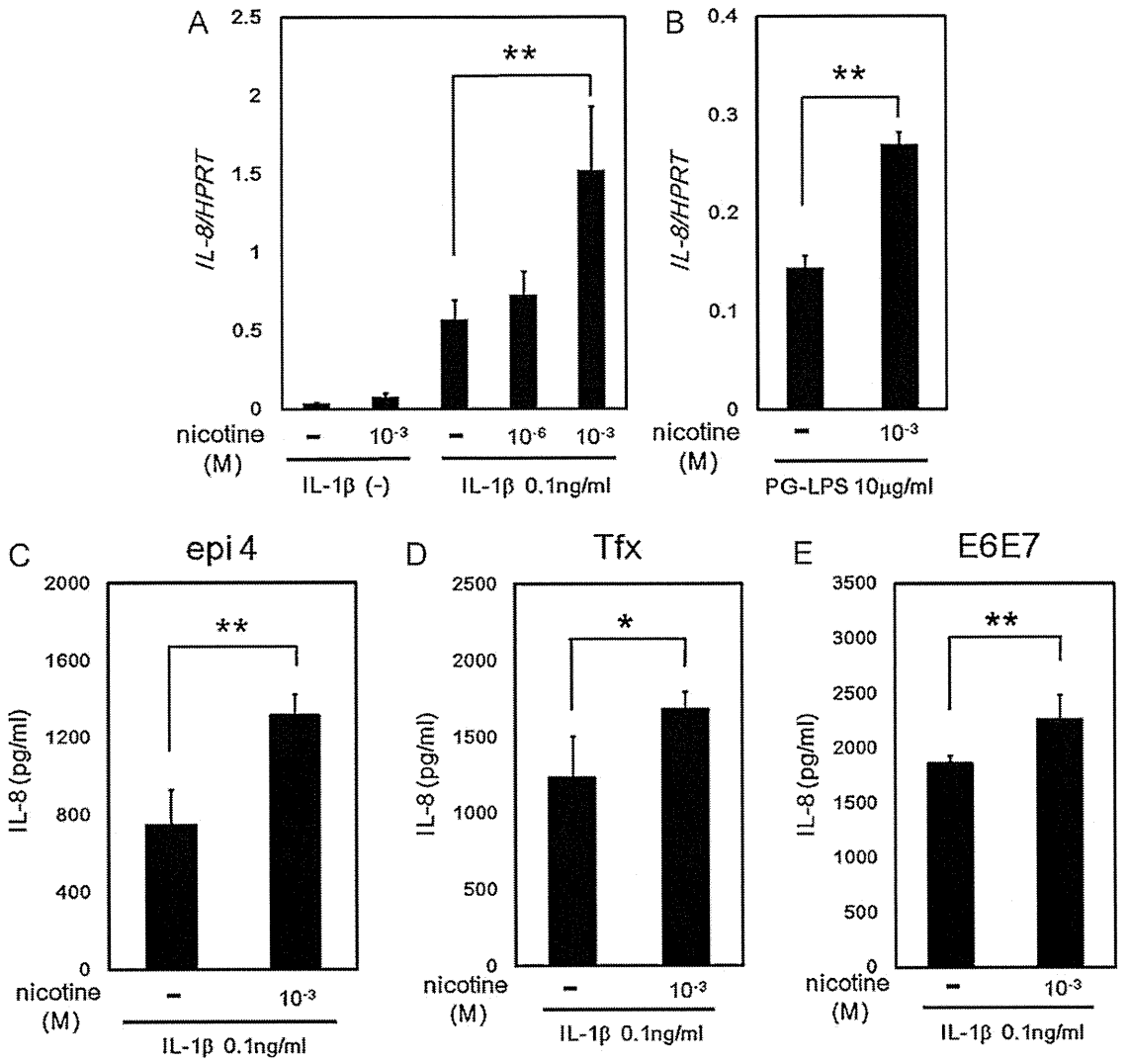

Fig. 2 – Nicotine up-regulates the expression of IL-8. IL-8 mRNA expression in epi 4 stimulated with IL-1β (A) or *P. gingivalis* LPS (B: PG-LPS) in the presence or absence of nicotine for 12 h was quantified using real-time PCR. Data are expressed as mean ± SD of three determinations. **$p < 0.01$ compared 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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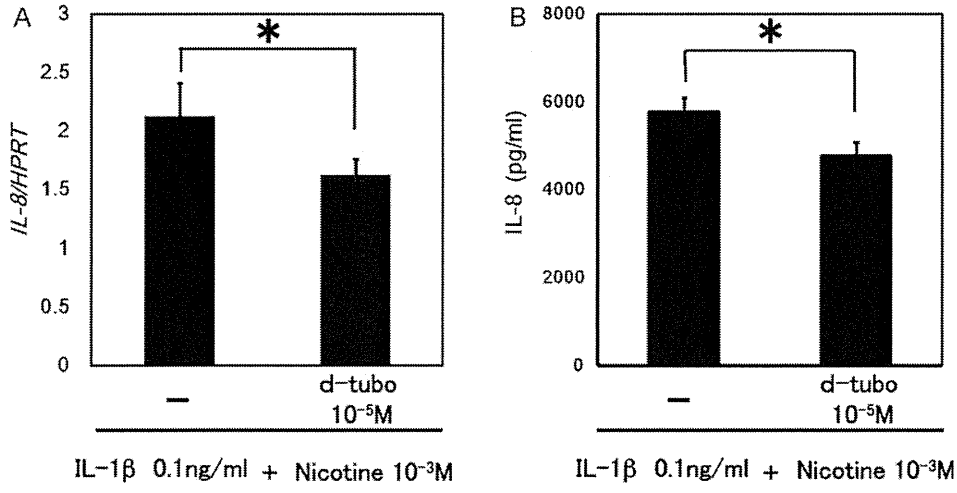

Fig. 3 – d-Tubocurarine, a nonselective nAChR antagonist, inhibits nicotine-induced upregulation of IL-8. After pretreatment with d-tubocurarine for 1 h, epi 4 was stimulated with IL-1β and nicotine for 12 h (A: real-time PCR) or 24 h (B: ELISA). Data are expressed as mean ± SD of three determinations.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

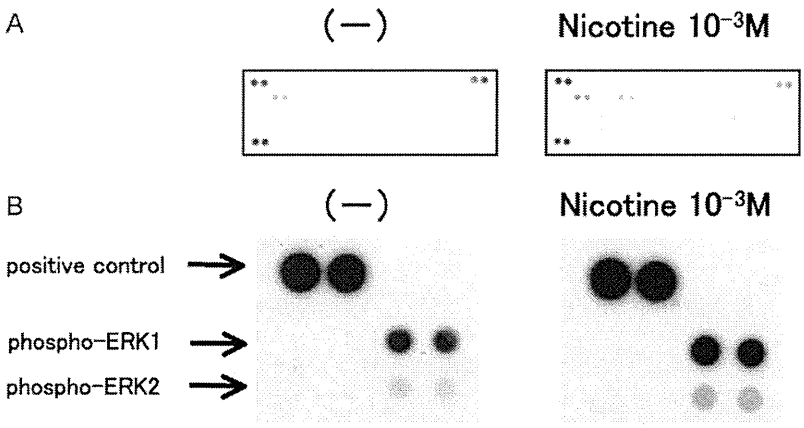

Fig. 4 – Investigation of MAPK phosphorylation in nicotine-stimulated epi4 cells. epi 4 cells were cultured in the presence or absence of nicotine for 10 min. To investigate phosphorylation of 21 MAPKs, a Human Phospho-MAPK Array Kit™ was utilized. All data (A), the enlargement of the area blotted for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

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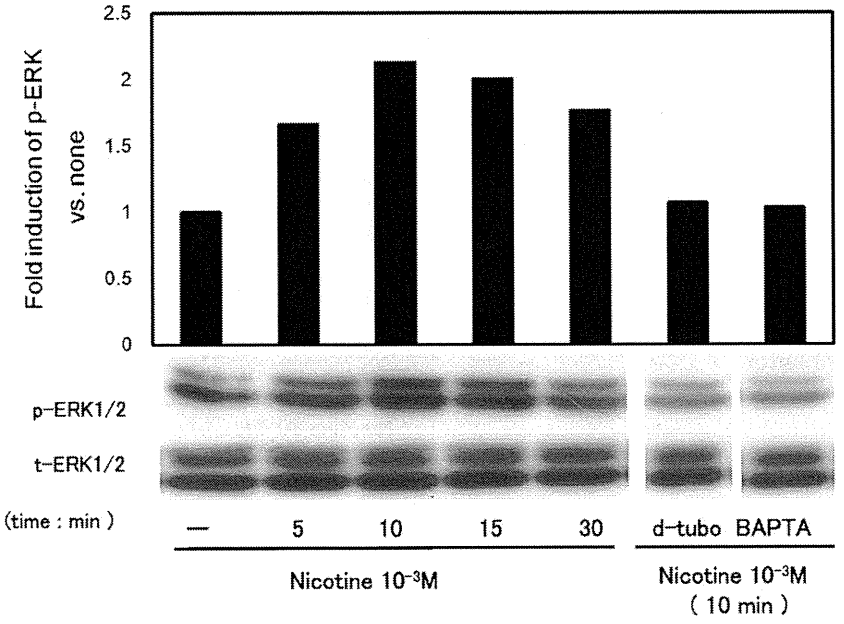

Fig. 5 – d-Tubocurarine and BAPTA-AM inhibit nicotine-induced upregulation of ERK1/2 phosphorylation. After pretreatment with d-tubocurarine or BAPTA-AM for 1 h, epi 4 cells were stimulated with nicotine for 10 min. Cell lysate was subjected to immunoblotting for p-ERK. Blotting images were analysed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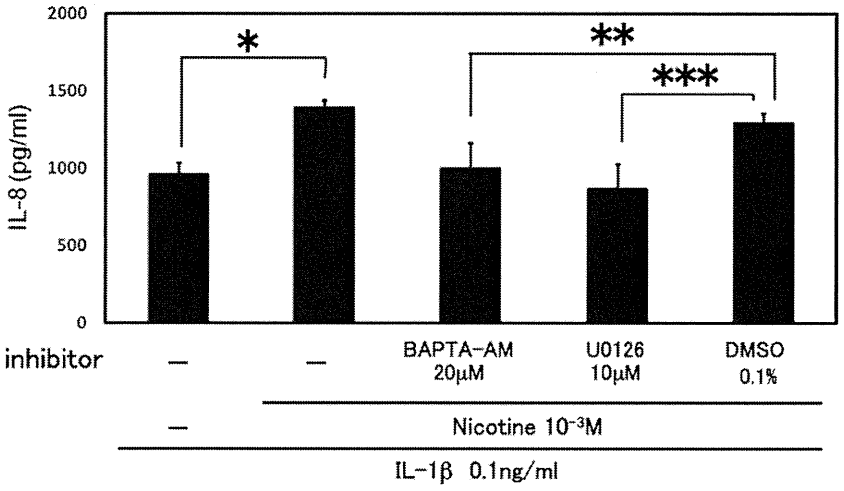

Fig. 6 – BAPTA-AM and U0126 inhibit nicotine-induced up-regulation of IL-8 production. After pretreatment with BAPTA-AM and U0126 for 1 h, epi 4 cells were stimulated with IL-1β in the presence or absence of nicotine for 24 h. The concentration of IL-8 was evaluated using ELISA. Data are expressed as 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

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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

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γ gene in macrophages caused an upregulation of inflammatory cytokine production [30]. In addition, PPAR γ regulated the maturation and function of DC [31–34]. These findings suggest that PPAR γ plays an important role in inflammation and immunity.

In the present study, we investigated the effects of nicotine on the differentiation of human monocytes into DCs. Our results demonstrate that DCs differentiated in the presence of nicotine (NiDCs) reduce inflammatory cytokine production and induce the expression of coinhibitory molecules compared to those in the absence of nicotine (MoDCs). Furthermore, LPS-stimulated NiDCs induce differentiation of naive CD4 T cells into Th2 cells, whereas LPS-stimulated MoDCs induce Th1 immune responses. NiDCs are also associated with increased expression of PPAR γ and PPAR γ-target genes. Finally, our study suggests that nicotine modulates the DC phenotype by upregulation of PPAR γ gene expression.

2. Materials and methods

2.1. Isolation of monocytes, and generation of monocyte-derived dendritic cells

All human subjects participating in this study after provided informed consent to a protocol that was reviewed and approved by the Osaka University Graduate School of Dentistry Institutional Review Board. Peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers, and monocytes were isolated by standard density gradient centrifugation using Histo-Paque 1077 (Sigma–Aldrich, St. Louis, MO), followed by anti-CD14 microbeads magnetic cell sorting, and processed according to the manufacturer's instruction (Miltenyi Biotec, Auburn, CA). The purity of the CD14 positive monocytes was >95%. Control DC (-nicotine) or test DC ($+10^{-8}$ to 10^{-2} M nicotine) were generated in complete RPMI-10 (RPMI-1640 with a final concentration of 10% heat-inactivated FCS, 20 mM Hepes, 50 μg/ml gentamicin) supplemented with 25 ng/ml, IL-4 (R&D Systems, Minneapolis, MN) and 50 ng/ml GM-CSF (R&D Systems). Non-adherent cells were harvested on day 6 or 7. Nicotine (Sigma–Aldrich) was prepared in PBS and neutralized to pH 7.2. In some experiments, monocytes were pretreated for 30 min in the presence of the non-selective and competitive nAChR antagonist, d-tubocurarine (Sigma–Aldrich) prior to supplementation with IL-4 and GM-CSF. $CD45RA^+$ and $CD4^+$ naive T cells were obtained from PBMC isolation of CD4 T cells using a Naive $CD4^+$ T cells Isolation kit (Miltenyi Biotec).

2.2. Analysis of DC surface molecules by flow cytometry

Expression of cell surface molecules was evaluated by flow cytometry. Immature DCs with or without nicotine (NiDCs and MoDCs, respectively) were cultured with 10 ng/ml lipopolysaccharide (LPS; *Salmonella minnesota*; List Biological Laboratories, INC, Campbell, CA) to induce cytokine and chemokine production. After 48 h, cells were harvested and incubated at 4 °C in the dark for 30 min with mAbs at 5 μg/ml or isotype-matched control Abs. FITC-conjugated Abs (BD Biosciences, San Jose, CA, unless noted) used for the experiments were anti-CD14, anti-HLA-DR, anti-CD40, anti-CD80, and anti-CD86. PE-conjugated Abs used for the experiments were anti-CD1a, anti-PD-L1, anti-PD-L2, anti-ILT3 (Beckman Coulter, Marseille, France) and anti-ILT4 (Beckman Coulter). Cells were washed twice and data were acquired on a FACSCalibur (BD Biosciences). Data from viable cells were analyzed with CELLQuest™ software (BD Biosciences).

2.3. Antigen uptake by DCs

MoDCs or NiDCs were washed with PBS and suspended in complete RPMI-10 containing FITC-dextran (200 μg/ml: Molecular Probes, Eugene, OR). After 60 min-incubation at 37 °C or 4 °C (as negative control), cells were washed three times, resuspended with PBS, and analyzed by flow cytometry.

2.4. Allogeneic T cell proliferation

MoDCs and NiDCs were stimulated with 10 ng/ml LPS for 48 h, treated with mitomycin C (50 μg/ml for 1 h) to inhibit DC proliferation, and then co-cultured with 10^5 naive $CD4^+$ T cells for 6 days. Allogeneic T cell proliferation was measured using the non-radioactive colorimetric assay WST-1 system (Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer's instructions and the OD450/650 measured after 2 h on a micro plate reader (Bio-Rad, Hercules, CA).

2.5. Measurement of cytokine secretion

MoDCs and NiDCs were stimulated with 10 ng/ml LPS for 48 h, and supernatants were frozen at −80 °C until used for measurement of cytokine secretion. Supernatants cytokine levels were determined using IL-12 (p40 + p70), IL-10 and TNF-α ELISA kits (Pierce Endogen, Rockford, IL). To measure cytokine production by T cells, 10^5 naive $CD4^+$ T cells were co-cultured for 6 days with 10^4 unstimulated DCs or LPS-stimulated DCs treated with mitomycin C. After 6 days culture, cells were restimulated at 2×10^5 cells/well with plate-bound anti-CD3 (eBioscience) and soluble anti-CD28 (eBioscience) for 24 h. Supernatants were frozen at −80 °C until use. Cytokine levels were determined in supernatants using IFN-γ, IL-5, and IL-10 ELISA kits (Pierce Endogen).

2.6. RT-PCR assay and real-time quantitative RT-PCR assay

Total RNA was extracted from cells using the RNAbee kit (TEL-TEST, Friendswood, TX) according to the manufacture's instructions. cDNA synthesis and amplification via PCR were performed as previously described. HPRT (hypoxanthine phosphoribosyl transferase) was used as a positive control for RNA integrity. After denaturation at 95 °C for 5 min, each cycle consisted of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. Amplified products were analyzed by electrophoresis at 100 V for 30 min on 1.5% TAE agarose gels containing 0.5 mg/ml ethidium bromide. Band density was quantified with Quantity One software (Bio-Rad, Hercules, CA). Quantitative real-time PCR was performed with an ABI7700 system (Applied Biosystems, Tokyo, Japan) using the following primers;

PPAR γ (forward, 5′-TGGAATTAGATGACAGCGACTTGG-3′: reverse, 5′-CTGGAGCAGCTTGGCAAACA-3′).
CD1a (forward, 5′-TCGGGTGAAGCACAGCAGTC-3′: reverse, 5′-GGCACTATCACCGCCAAGATG-3′).
Adipose differentiation-related protein: ADFP (forward, 5′-CGGATGATGCAGCTCGTGA-3′: reverse, 5′-GCACGGGAGTGAAGCTTGGTA-3′).
Apolipoprotein E: ApoE (forward, 5′-CTGCGTTGCTGGTCACATTC-3′: reverse, 5′-CTCCTGCACCTGCTCAGACA-3′).
Fatty-acid-binding protein-4: FABP4 (forward, 5′-CTTCATACTGGGCCAGGAATTTG-3′: reverse, 5′-CTCCTGCACCTGCTCAGACA-3′).
Hypoxanthine phosphoribosyl transferase: HPRT (forward, 5′-GGCAGTATAATCCAAAGATGGTCAA-3′: reverse, 5′-GTCAAGGGCATATCCTACAACAAAC-3′). HPRT served as a housekeeping gene.

2.7. Measurement of PPAR γ activation

PPAR γ activation in nuclear extracts was determined by TransAM PPAR γ kit (Active Motif Inc., Carlsbad, CA) according to

the manufacture's protocol. In brief, 5 μg of nuclear extract was incubated for 1 h in a 96-well plate immobilized with an oligonucleotide containing PPAR γ binding site. Antibody was added and incubated for 1 h. Anti-IgG horseradish peroxidase was added and incubated for an additional 1 h. Plates were washed and developing solution added, followed by stop solution, and the OD450/650 measured on a micro plate reader (Bio-Rad).

2.8. Statistical analysis

Data were expressed as the mean ± SD. Statistical analysis of the results was performed with Student's *t* test or ANOVA followed Dunnett multiple comparison test. Differences were considered statistically significant when *p* value were less than 0.05.

3. Results

3.1. Effect of nicotine on differentiation of monocytes into DCs

Monocyte can differentiate into DCs in the presence of IL-4 and GM-CSF. The addition of IL-4 and GM-CSF to cells when they are initially cultured will lead to upregulation of CD1a expression and downregulation of CD14 expression. Different concentrations of nicotine were added with IL-4 and GM-CSF to determine the possible effect of nicotine on differentiation. FACS acquisition of cell surface expression data was obtained on day 7. As shown in Fig. 1A, MoDCs without nicotine presented the typical phenotype of monocyte-derived DCs characterized by high CD1a expression and low level CD14 expression (data not shown). Of the different nicotine concentrations tested, 10^{-3} M reduced CD1a expression. In preliminary experiments, we confirmed that nicotine (10^{-8} to 10^{-3} M) did not affect DC viability as indicated by trypan blue exclusion and WST-1 assays. The cell viabilities with or without 10^{-3} M nicotine were 29.1% and 30.7% by trypan blue exclusion, and 34.0% and 35.3% by WST-1 assay, respectively.

3.2. The pattern of cytokine production is altered by nicotine

DCs produce several cytokines and chemokines depending on the extracellular environment and stimuli. Recent studies have shown that CD1a is a marker for DC production of IL-12 and Th1 polarization [35,36]. As shown in Fig. 1A, nicotine reduced the expression of CD1a in DCs, whereas the effect of nicotine on Th1/2 polarization remained unclear [23–25]. We were interested in whether DCs differentiated in the presence of nicotine would produce Th1/Th2 cytokines. We therefore examined the production of IL-12 (p40 + p70), IL-10, and TNF-α. In this experiment, DCs were activated in the presence of LPS, which augments the Th1 response. Supernatants of non-stimulated and LPS-stimulated MoDCs and NiDCs were assayed for IL-12 (p40 + p70), IL-10 and TNF-α. IL-12 (p40 + p70) and TNF-α production by NiDCs after LPS stimulation was significantly reduced (Fig. 1B). Since IL-10 production of LPS-stimulated NiDCs was also reduced, it is unlikely that inhibition of IL-12 and TNF-α secretion in the presence of nicotine was mediated through IL-10, an anti-inflammatory cytokine. These results suggest that nicotine may impair Th1 polarization.

3.3. NiDCs show impaired T cell proliferation

The ability of MoDCs and NiDCs to cause proliferation of allogeneic naive T cells was compared. MoDCs and NiDCs were cultured with or without LPS, harvested after 48 h, and co-cultured with naive T cells for 6 days. As shown in Fig. 2A, MoDCs and NiDCs in the presence of LPS resulted in significant T cell proliferation compared to MoDCs and NiDCs without LPS. Interestingly, however, there was significant reduction of T cell proliferation when cultured with LPS-stimulated NiDCs compared to LPS-stimulated MoDCs. These results suggest that the ability of antigen-presentation by DC to stimulate allogeneic T cells is diminished following nicotine treatment.

3.4. Cytokine-secretion profile of $CD4^+$ T cells primed with DCs developed with or without nicotine

To further characterize the effect of nicotine on the priming capacity of DCs, the expanded T cells were restimulated with anti-CD3 and anti-CD28. Supernatants were collected and levels of IFN-γ, IL-5, and IL-10 were measured. The results summarized in Fig. 2B showed that $CD4^+$ T cells cultured with MoDCs produced elevated IFN-γ and decreased levels of IL-10. $CD4^+$ T cells cultured

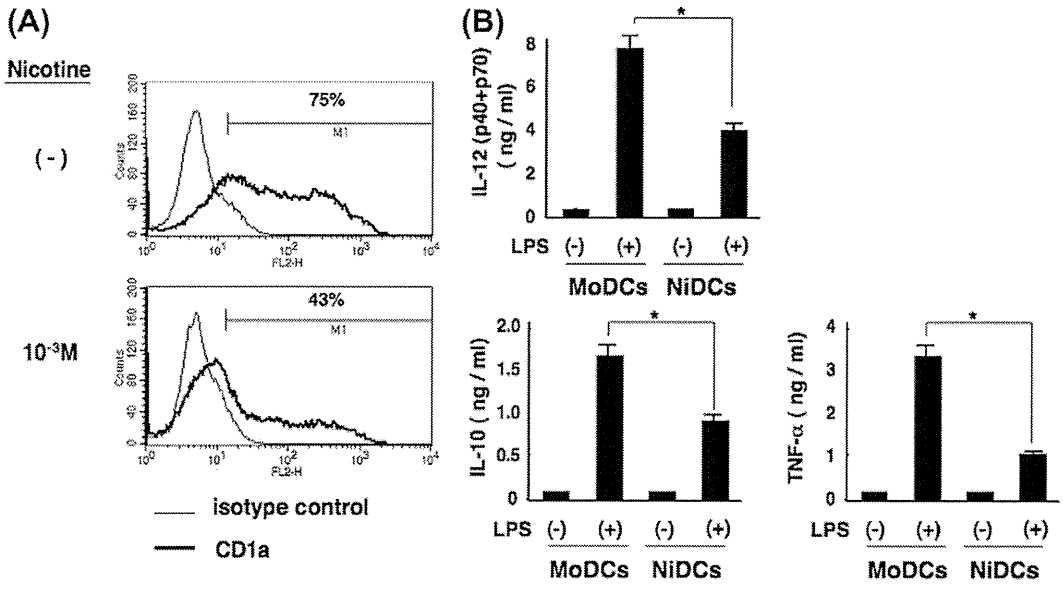

Fig. 1. (A) Differentiation of $CD1a^+$ DC from monocytes in the presence (NiDCs) or absence (MoDCs) of 1×10^{-3}M nicotine. MoDCs and NiDCs were harvested at day 7 and analyzed by FACS for the expression of $CD1a^+$. These data represent one of eight independent experiments with monocytes isolated from different donors. (B) Cytokine production by MoDCs and NiDCs. MoDCs and NiDCs were cultured in the absence or presence of 10 ng/ml LPS for 24 h. Supernatants were tested for cytokine secretion by ELISA. Results are shown as mean values ± SD of at least five independent experiments. *$P < 0.05$ compared with LPS-stimulated MoDCs.

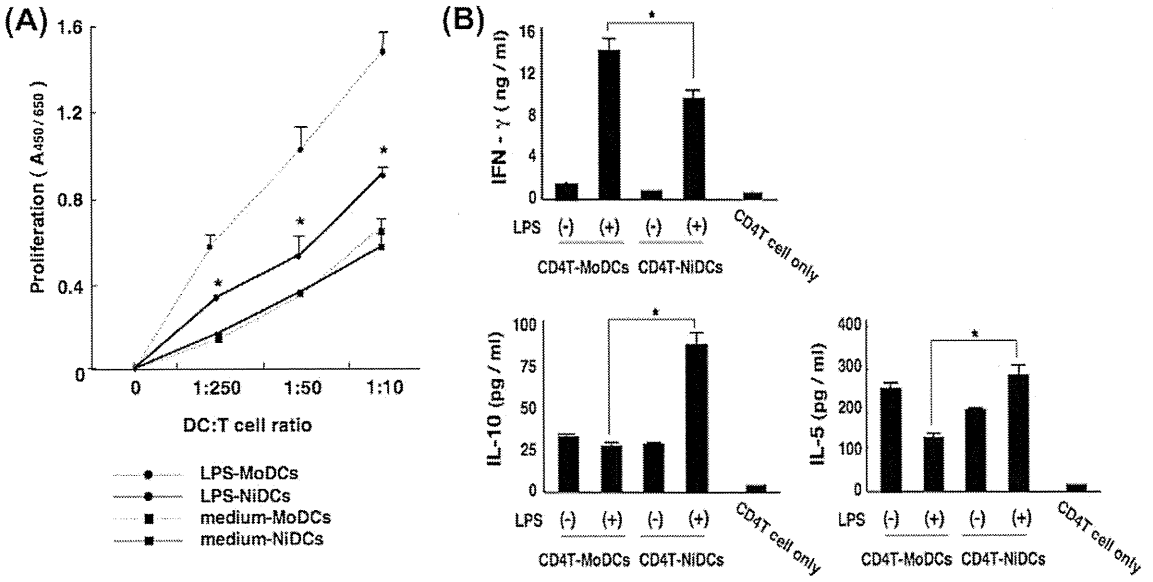

Fig. 2. Effect of nicotine on antigen-presenting properties of DC and cytokine production in MLR. (A) Comparisons of the effects of MoDCs and NiDCs on T cell proliferation. The results represent the mean OD values ± SD obtained from triplicate cultures. The data shown were obtained from one of five independent experiments. *$P < 0.05$ compared with LPS-stimulated MoDCs. (B) Supernatants of restimulated T cells were harvested and measured for IFN-γ, IL-5 and IL-10 levels by ELISA. The results represent the mean values ± SD obtained from triplicate cultures. These data represent one of five independent experiments. *$P < 0.05$ compared with LPS-stimulated MoDCs.

with NiDCs produced elevated levels of IL-5 and IL-10 relative to control $CD4^+$ T cells (MoDCs group). These results suggest that nicotine has the ability to differentiate naive T cells into Th2 $CD4^+$ T cells. We tested for additional Th2 cytokine (IL-4 and IL-13) by ELISA, but neither was detected.

3.5. Nicotine altered coinhibitory/costimulatory molecule expression

As shown in Fig. 2A, T cell proliferation was reduced in the presence of NiDCs. Although we assessed antigen uptake by MoDCs and NiDCs, no difference was observed between the two cell types (data not shown). To investigate the differences in T cell stimulation mediated by MoDCs and NiDCs, we analyzed expression levels of HLA-DR, CD40, B7 costimulatory molecules (CD80, CD86), coinhibitory molecules [the programmed cell death ligand (PD-L)1 and PD-L2] and inhibitory receptors [the immunoglobulin-like transcripts (ILT)3 and ILT4] on MoDCs and NiDCs after activation with LPS. In the absence of LPS, expression levels did not differ between MoDCs and NiDCs. Following LPS stimulation, NiDCs showed significantly elevated levels of PD-L1 and ILT4 when compared with MoDCs (Fig. 3C and D). The expression of CD86 and PD-L2 on LPS-stimulated NiDCs was slightly elevated in average but not significantly compared to LPS-stimulated MoDCs. Interestingly, ILT3 expression on NiDCs was not changed after LPS stimulation whereas MoDCs showed a reduction (Fig. 3D). The expression of HLA-DR, CD40, and CD80 on NiDCs was not significantly different from those on MoDCs following LPS stimulation (Fig. 3A and B).

3.6. PPAR γ expression in NiDCs

Recent studies have shown that PPAR γ is a potential regulator of antigen-presenting cells and T cells. NiDCs showed a reduction in inflammatory cytokine production, and had a lower capacity to induce T cell proliferation, and Th2 polarization. Characteristics of NiDCs were similar to those of PPAR γ agonist-treated MoDCs. Therefore, we examined PPAR γ expression in MoDCs and NiDCs. We observed increased expression of PPAR γ mRNA in NiDCs (Fig. 4A and B). We then measured PPAR γ activity in nuclear extracts from MoDCs and NiDCs using a TransAM PPAR γ ELISA kit. Results indicate that PPAR γ activity in NiDCs was significantly higher than in MoDCs (Fig. 4C). Since PPAR γ was induced in NiDCs, we further investigated expression of adipose differentiation-related protein (ADRP), apolipoprotein E (ApoE), and fatty-acid-binding protein-4 (FABP4), which are known target genes of PPAR γ. As shown in Fig. 4D, upregulation of these three genes occurred only in NiDCs and correlated directly to PPAR γ expression.

3.7. Effect of non-selective nAChR antagonist on DC differentiation in the presence of nicotine

To examine whether effect of nicotine on DC development is mediated by nicotinic acetylcholine receptors (nAChRs), monocytes were preincubated with the non-selective and competitive nAChR antagonist, d-tubocurarine (1 μM) 30 min before culture. As shown in Fig. 5A, CD1a expression was recovered by pretreatment of cells with d-tubocurarine. These data indicates that the effect of nicotine on CD1a expression depends mainly on specific interaction with nAChRs. In addition, the effect of nicotine on the induction of PPAR-γ, ADFP, and ApoE gene expressions was clearly inhibited by pretreatment with d-tubocurarine (Fig. 5B–D).

3.8. Blocking nAChRs recovered Th1 response

As shown in Fig. 6, inhibition of nicotine signal by pretreatment with d-tubocurarine (1 μM), caused inhibition of PPAR γ expression (Fig. 6). Thus, we investigated whether d-tubocurarine-treated NiDCs recovered Th1 responses. As shown in Fig. 6, pretreatment of d-tubocurarine reversed reduction of IL-12 (p40 + p70) secretion by NiDCs (Fig. 6A) and IFN-γ secretion by T cells cocultured with LPS-activated NiDCs (Fig. 6B).

4. Discussion

Nicotine and cigarette smoke extracts containing nicotine are reported to have immuno-modulating effects in human and mouse [23–25,37–39]. Nicotine is a major chemical component of cigarette smoke that contains 3000–4000 chemical compounds. We tested our hypothesis that nicotine is one of the main causes of