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

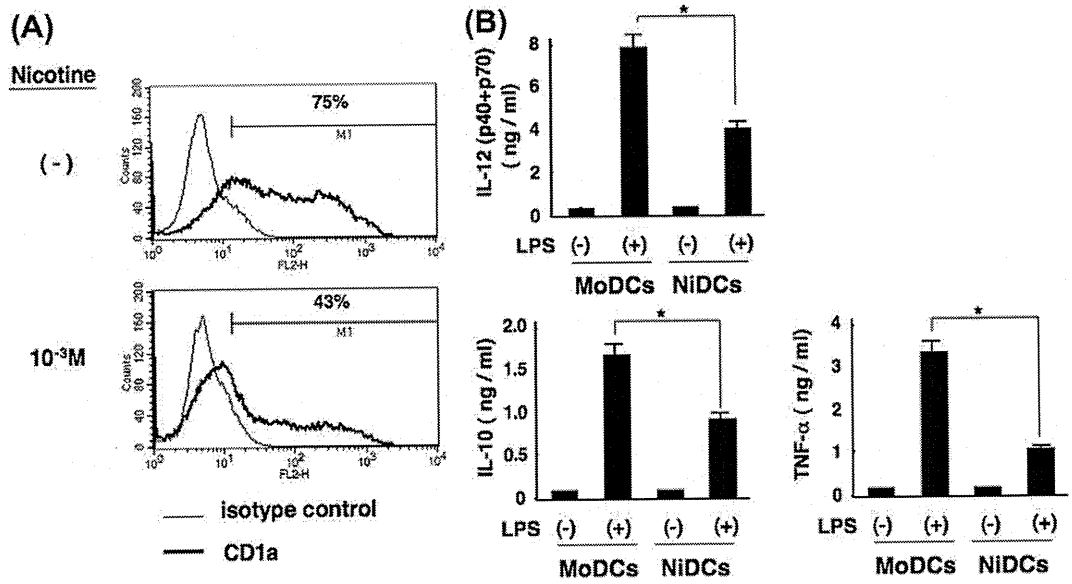

Fig. 1. (A) Differentiation of $CD1a^+$ DC from monocytes in the presence (NiDCs) or absence (MoDCs) of 1×10^{-3}M nicotine. MoDCs and NiDCs were harvested at day 7 and analyzed by FACS for the expression of $CD1a^+$. These data represent one of eight independent experiments with monocytes isolated from 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.

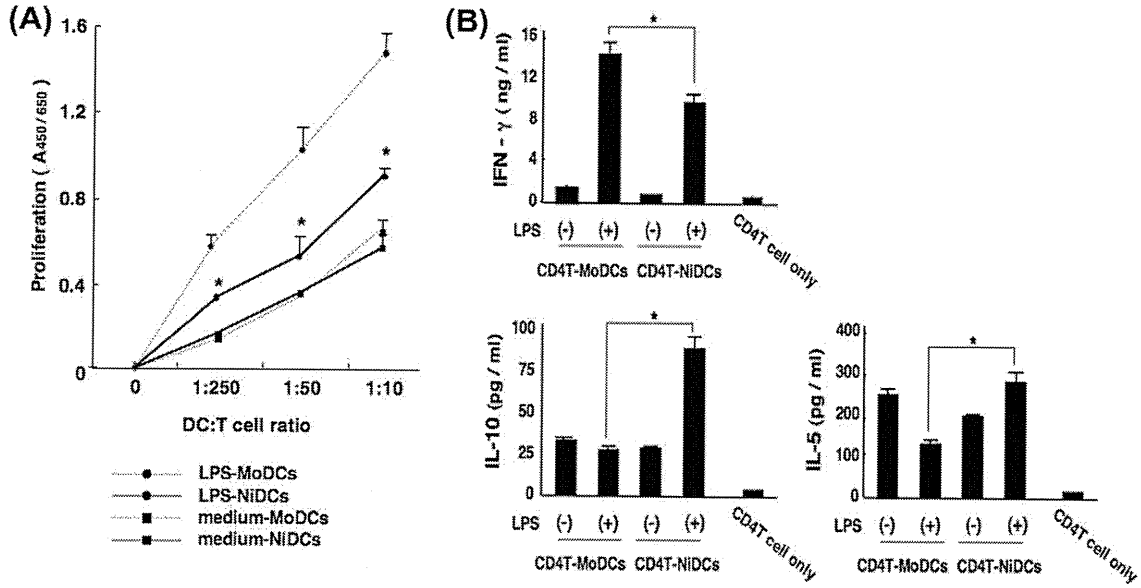

Fig. 2. Effect of nicotine on antigen-presenting properties of DC and cytokine production in MLR. (A) Comparisons of the effects of MoDCs and NiDCs on T cell proliferation. The results represent the mean OD values ± SD obtained from triplicate cultures. The data shown were obtained from one of five independent 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

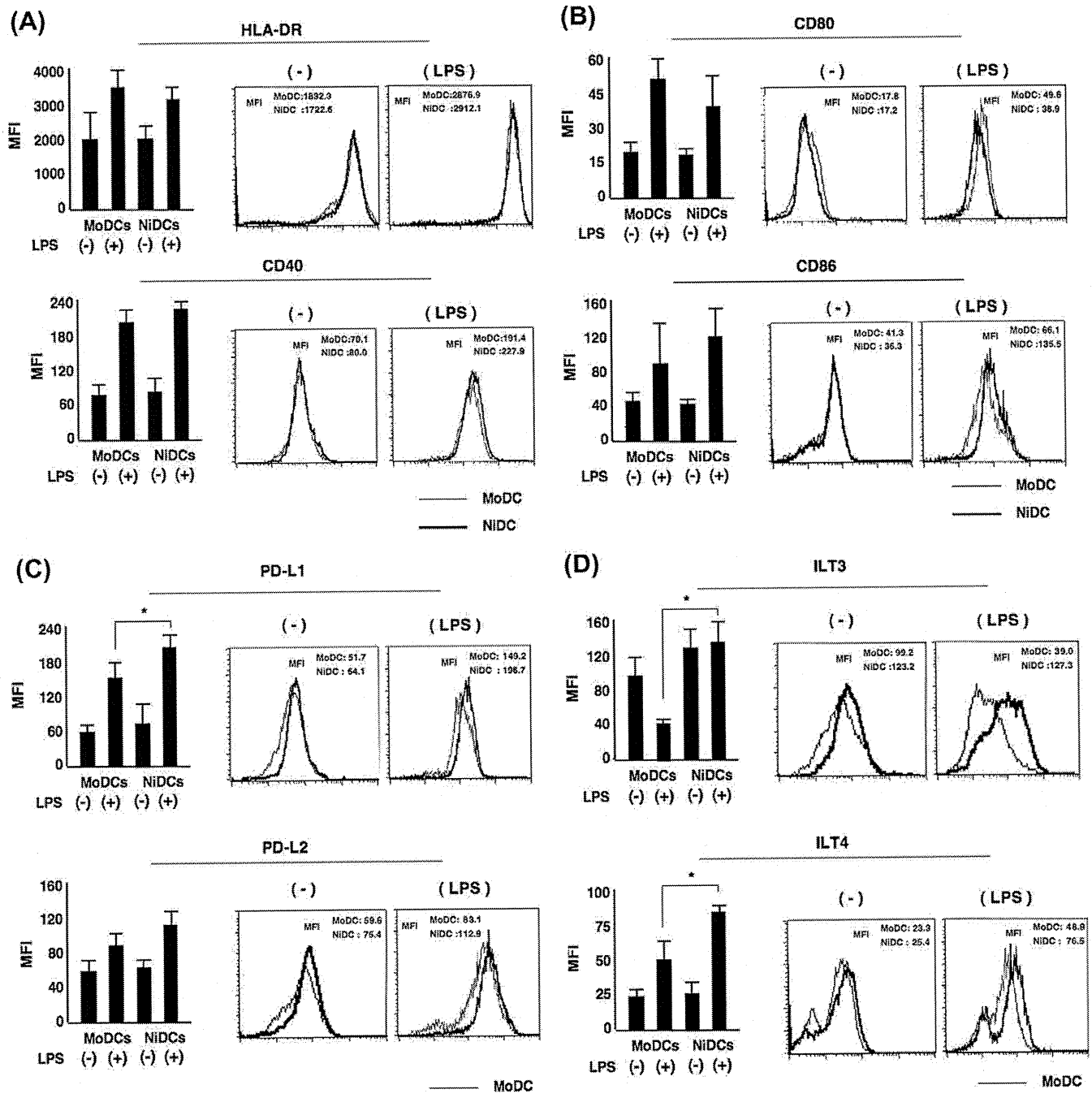

Fig. 3. Effect of nicotine on the expression of costimulatory/coinhibitory molecules on DCs. Expression of HLA-DR(A), CD40(A), CD80(B), CD86(B), PD-L1(C), PD-L2(C), ILT3(D) and ILT4 (D) on MoDCs or NiDCs stimulated with LPS (10 ng/ml) for 48 h was evaluated by FACS. Left panels: bar graphs are shown as mean values ± SD of five independent experiments. *$P < 0.05$ when compared with MoDCs stimulated with LPS. MFI: mean fluorescent intensity. Right panels: one representative FACS histogram of MoDCs (thin line) and NiDCs (thick line) of more than six independent experiments are shown.

tobacco toxicity that alters immune function. Considering the many components of cigarette smoke, nicotine may not be responsible for all types of tobacco toxicity. The expression of nicotinic acetylcholine receptors, however, indicates that the effects of nicotine may be transmitted and mediated by a specific cellular signaling pathway(s) coupled to immunological function. Although 10^{-3} M nicotine is greater than the pharmacological dose, the concentration of nicotine in saliva of smokers may reach mM levels in localized areas such as the oral cavity and respiratory tract [14–16]. We therefore assumed that direct and local exposure of high doses of nicotine to the inflammatory lesion with bleeding in gingival tissue initiates monocytes to differentiate into DC in inflamed gingival microcirculation. In preliminary experiments, we used 10^{-8} to 10^{-2} M nicotine to investigate the effect of nicotine on DC phenotypic changes, and found that 10^{-3} M nicotine significantly induced DC characteristics. Additionally, we confirmed that 10^{-3} M nicotine did not impact DC viability. Therefore, we chose 10^{-3} M nicotine to induce differentiation of monocytes into DCs.

We report that *in vitro* differentiation of DCs from monocytes in the presence of nicotine yields a subset of DC (NiDCs) characterized by an altered phenotypic profile and modulated functions. CD1a is one of five members of the CD1 family and has been used as a DC biomarker [40]. There is however a significant heterogeneity in CD1a expression in DCs. Both CD1a (+ or −) DCs have been

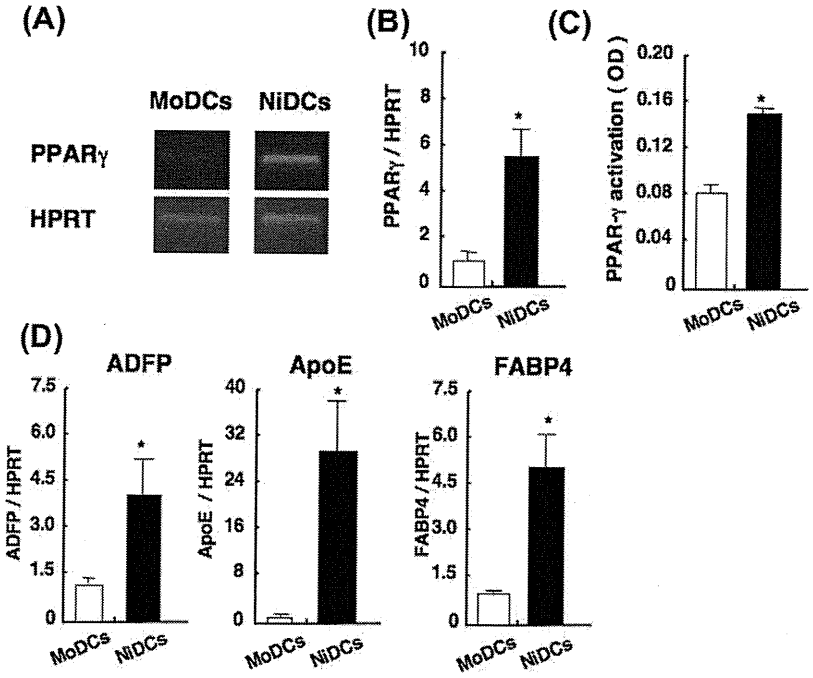

Fig. 4. Expression and activation of PPAR γ in MoDCs and NiDCs. (A) Representative RT-PCR analysis of PPAR γ mRNA expression. One representative profile of six performed. (B) Quantitative analyses of PPAR γ mRNA expression by real-time RT-PCR. mRNA levels are expressed as fold change above control mRNA (HPRT). Results are shown as mean values ± SD of five independent experiments. (C) Activation of PPAR γ in MoDCs and NiDCs. Nuclear extracts taken at a half-hour were analyzed by the TransAM PPAR γ assay. Results are shown as mean values ± SD of five independent experiments and are expressed as ratio of OD_{450}. (D) Expression of the PPAR γ target genes in MoDCs and NiDCs. Results are shown as mean values ± SD of five independent experiments. $^*P < 0.05$ when compared with MoDCs.

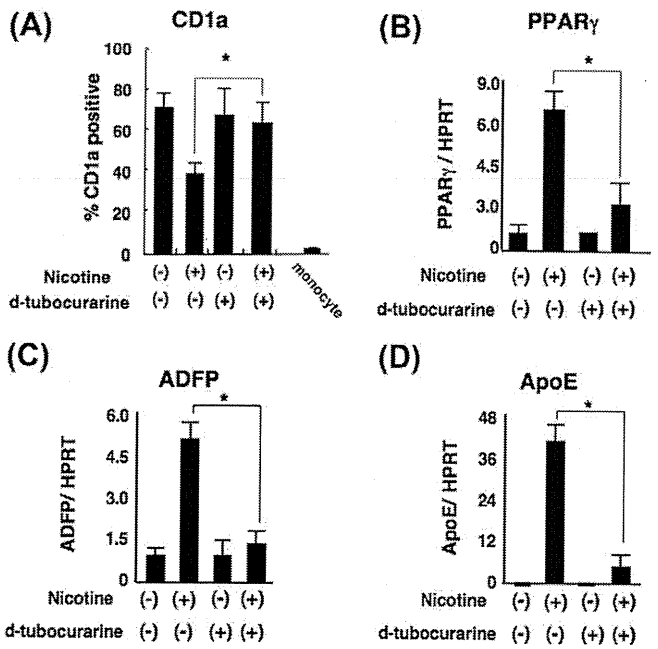

Fig. 5. Effect of d-tubocurarine, a non-selective nAChRs antagonist on mRNA expression of PPAR γ and PPAR γ target genes in MoDCs and NiDCs. (A) Expression of CD1a was examined at day 7 to evaluate the effect of nicotine and d-tubocurarine. Results are shown as mean values ± SD of five independent experiments. (B–D) Quantitative analyses of PPAR γ, ADFP, and ApoE mRNA expression by real-time RT-PCR. mRNA levels are expressed as fold change above control mRNA (HPRT). Results are shown as mean values ± SD of four independent experiments. $^*P < 0.05$ when compared with DCs differentiated in the presence of nicotine with pretreatment of d-tubocurarine.

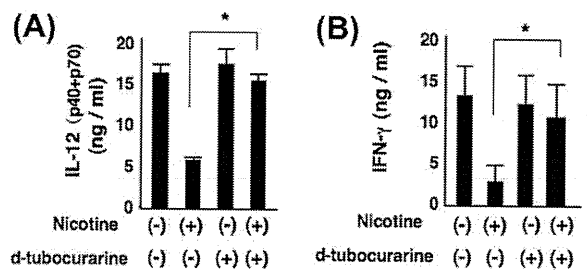

Fig. 6. Effect of d-tubocurarine, non-selective nAChRs antagonist on Th1 responses in MoDCs and NiDCs. (A) IL-12 (p40 + p70) production by MoDCs and NiDCs. MoDCs and NiDCs pretreated with d-tubocurarine (1 μM) were cultured in the presence of 10 ng/ml LPS for 48 h. Supernatants were then tested for IL-12 (p40 + p70) production by ELISA. (B) Supernatants of restimulated T cells were harvested and measured for IFN-γ level by ELISA. Results are shown as mean values ± SD of four independent experiments. $^*P < 0.05$ when compared with NiDCs without pretreatment of d-tubocurarine.

identified in peripheral blood [41]. In addition, recent studies suggest that the ratio of +/− forms of CD1a DCs differentiated from monocytes can be altered depending on the culture conditions [42–45]. In our study, a significant reduction of CD1a+ expression is observed in NiDCs when compared with MoDCs. The effect of nicotine on decreasing CD1a expression is inhibited by pretreatment of cells with d-tubocurarine, a non-selective nAChR antagonist. These results indicate that nicotine interaction with nAChRs can mediate DC differentiation.

As seen in Fig. 1B, LPS-stimulated NiDC secreted lower amounts of pro-inflammatory cytokines such as IL-12 (p40 + p70) and TNF-α, compared with MoDCs. These data confirm results from previous studies in which nicotine and cigarette smoke extracts (CSE) suppressed DC function, impaired antigen-presentation to induce naive T cell proliferation and alter Th1 responses into those seen in Th2 cells [23,24]. These studies also described that CSE suppressed IL-12 production in activated DCs, and diminished the effects of costimulatory molecules such as CD40 and CD80. We observed poor induction of T cell proliferation by NiDCs, elevation of the coinhibitory molecules PD-L1 and ILT4, and a slight

upregulation of PD-L2 in LPS-stimulated NiDCs. Furthermore, ILT3 expression was maintained in NiDC after LPS-stimulation, although expression on LPS-stimulated MoDCs was reduced. Increased PD-L1 and PD-L2 expression combined with positive costimulatory molecules such as CD86 induce an immunotolerogenic function in DCs [46–48]. In addition, high expression of ILT3 and ILT4 on DCs is associated with immunotolerogenic characteristics [49,50]. The reduction in priming capacity of NiDCs may result from induction of inhibitory cell surface receptors.

PPAR γ has been reported to mediate several DC functions. PPAR γ-activated DCs altered the differentiation of naive CD4 T cells into Th2 cells [32,51]. In addition, PPAR-γ agonists inhibited TLR-mediated DC activation by interfering with the NF-κB and MAP kinase pathway [52]. In a conditional PPAR γ knockout mouse study, PPAR γ-activated DCs induced naive T cell anergy [31]. Other studies suggest that PPAR γ plays an important role in induction and maintenance of natural and induced Treg cells [53,54]. A recent report described that activation of PPAR γ strongly enhanced the expression of B7H1 (also termed PD-L1) [55]. We show that expression of PPAR-γ gene and PPAR-γ target genes are upregulated in NiDCs. ApoE plays important roles in lipoprotein clearance and homeostasis. ADRP plays a role in lipid body formation and cross-presentation of phagocytosed antigens to $CD8^+$ T cells. FABP4 plays an important role in the regulation of insulin sensitivity. In addition, both ApoE and FABP4 deficiency lead to development of atherosclerosis. Among those molecules, ADRP is likely to have a role in DC antigen presenting function. However, there is no information at present that these genes are involved in T cell proliferation and differentiation. With regard to PPAR-γ expression in NiDCs, further studies are needed to investigate the possible role of nicotine in induction of Treg cells as our data suggests that nicotine may differentiate monocytes into tolerogenic DCs.

The molecular mechanism of elevated PPAR γ expression in NiDCs needs to be clarified. Results from recent studies indicate that α7nAChR is crucial to the regulation of systemic inflammation, and that nicotine and acetylcholine control inflammatory cytokine production from endotoxin-stimulated macrophages by inhibiting NF-κB pathway via α7nAChRs [11,13]. We demonstrate that pretreatment of DCs with d-tubocurarine, a non-selective nAChR antagonist, reversed inhibition of CD1a expression, IL-12 production and Th1 responses in the presence of nicotine. One point that appears to be clear is that nicotine and nicotinic agonists prevent endotoxin-induced activation of the NF-κB pathway and induce expression of PPAR γ. Upregulation of PPAR γ and downregulation of NF-κB may synergistically induce the NiDC phenotype described previously. Blocking nAChRs by antagonist recovered Th1 response, however, there was no significant difference in IL-5 and IL-10 production between antagonist-treated NiDCs and control-NiDCs (data not shown). Presumably, the pathway inducing Th2 immune responses may be influenced by endocytosed nicotine, and not only by nicotine acting via nAChRs signaling.

Cigarette smoking leads to many health problems worldwide by significantly increasing the risk of diseases such as lung cancer, cardiovascular disease, COPD, rheumatoid arthritis and periodontal disease [1–5,56–58]. Smoking-related suppression of immune responses includes reduction of NK activity [59], and inhibition of microbicidal activity of macrophages [8,60]. Cigarette smoking and nicotine, however, may actually reduce severe inflammation in patients with ulcerative colitis (UC) [61,62]. In an animal study, conditional deletion of the PPAR γ-encoded gene in intestinal epithelial cells or macrophages caused an exacerbation of experimental colitis [30,53]. In addition, PPAR γ expression in the colonic mucosa is impaired in UC patients [63]. Results from these studies in addition to results from our study, indicate that nicotine signaling may induce expression and activation of PPAR γ.

In conclusion, our study provides new data indicating that nicotine reduces inflammatory cytokine production, and suppresses T cell priming capacity of DC via nAChRs. The suggested mechanism is nicotinic upregulation of coinhibitory molecules. The effect of nicotine may be mediated by PPAR γ expression. These studies suggest a link between the effects of nicotine on DC function and smoking-related diseases. Understanding the immuno-modulatory effects of nicotine will provide new and useful information for the prevention of smoking-related diseases.

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640

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Adiponectin regulates functions of gingival fibroblasts and periodontal ligament cells

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Iwayama T, Yanagita M, Mori K, Sawada K, Ozasa M, Kubota M, Miki K, Kojima Y, Takedachi M, Kitamura M, Shimabukuro Y, Hashikawa T, Murakami S. Adiponectin regulates functions of gingival fibroblasts and periodontal ligament cells. J Periodont Res 2012; doi: 10.1111/j.1600-0765.2012.01467.x. © 2012 John Wiley & Sons A/S

Background and Objective: Adiponectin is a cytokine constitutively produced by adipocytes and exhibits multiple biological functions by targeting various cell types. However, the effects of adiponectin on primary gingival fibroblasts and periodontal ligament cells are still unexplored. Therefore, we investigated the effects of adiponectin on gingival fibroblasts and periodontal ligament cells.

Material and Methods: The expression of adiponectin receptors (AdipoR1 and AdipoR2) on human gingival fibroblasts (HGFs), mouse gingival fibroblasts (MGFs) and human periodontal ligament (HPDL) cells was examined using RT-PCR and western blotting. HGFs and MGFs were stimulated with interleukin (IL)-1β in the presence or absence of adiponectin, and the expression of IL-6 and IL-8 at both mRNA and protein levels was measured by real-time PCR and ELISA, respectively. Furthermore, small interfering RNAs (siRNAs) in MGFs were used to knock down the expression of mouse AdipoR1 and AdipoR2. The effects of adiponectin on the expression of alkaline phosphatase (*ALP*) and runt-related transcription factor 2 (*Runx2*) genes were evaluated by real-time PCR. Mineralized nodule formation of adiponectin-treated HPDL cells was revealed by Alizarin Red staining.

Results: AdipoR1 and AdipoR2 were expressed constitutively in HGFs, MGFs and HPDL cells. Adiponectin decreased the expression of IL-6 and IL-8 in IL-1β-stimulated HGFs and MGFs. AdipoR1 siRNA in MGFs revealed that the effect of adiponectin on reduction of IL-6 expression was potentially mediated via AdipoR1. Adiponectin-treated HPDL cells promoted the expression of *ALP* and *Runx2* mRNAs and up-regulated ALP activity. Furthermore, adiponectin enhanced mineralized nodule formation of HPDL cells.

Conclusion: Our observations demonstrate that adiponectin exerts anti-inflammatory effects on HGFs and MGFs, and promotes the activities of osteoblastogenesis of HPDL cells. We conclude that adiponectin has potent beneficial functions to maintain the homeostasis of periodontal health, improve periodontal lesions, and contribute to wound healing and tissue regeneration.

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Periodontal disease is a chronic inflammatory disease initiated by the biofilm of periodontopathic bacteria, leading to the destruction of periodontal tissues. Periodontal disease can be exacerbated by many factors;

for example, systemic diseases, such as diabetes, osteoporosis and immunodeficiency diseases, have been shown to result in an increased risk for periodontal disease (1). Interestingly, recent epidemiologic studies have suggested that obesity is also a risk factor for periodontitis (2,3). Obesity, which induces insulin resistance following systemic chronic inflammation (4), is one of the leading causes of type 2 diabetes, which is closely associated with periodontal diseases. Thus, it is possible that obesity and metabolic syndrome could be risk factors for the progression of periodontal diseases; however, the precise mechanism of how obesity results in the destruction of periodontal tissue remains unclear.

Adipokines, secreted by adipose tissue, can influence insulin resistance, inflammation and the cardiovascular system (5). Adiponectin – an adipokine – circulates in high concentrations in plasma (6). Two adiponectin receptors (AdipoRs) have been reported to be expressed on various tissues and cells (7). Importantly, hypo-adiponectinemia has been observed in patients with type 2 diabetes mellitus, obesity and coronary artery disease (8,9). Physiological concentrations of adiponectin suppressed tumor necrosis factor-α-induced inflammatory responses in human endothelial cells and macrophages (10). Recent studies have revealed that the concentrations of adiponectin in serum of patients with severe periodontitis are lower than those in serum from healthy subjects (11,12). Periodontal treatment has also been shown to increase the levels of adiponectin in chronic periodontitis (13). Interestingly, Yamaguchi *et al.* (14) reported that the levels of expression of AdipoRs were decreased in sites of severe periodontitis. These data suggest that adiponectin is involved in the homeostasis of periodontal tissues and may modulate inflammatory responses at periodontal lesions.

In recent years, adiponectin and AdipoRs have been reported to be expressed in osteoblasts (15,16), suggesting that adiponectin may be involved not only in anti-inflammatory functions but also in bone metabolism. Among periodontal tissues, periodontal ligament (PDL) cells have the potential to regulate neogenesis of alveolar bone and cementum and play important roles in events of wound healing and regeneration following periodontal tissue breakdown caused by progression of periodontal diseases. Considering the multifunctional role of adiponectin, adiponectin may affect the functional characteristics of PDL cells, which can differentiate into mineralized tissue-forming cells such as osteoblasts and cementoblasts (17).

In this study we investigated the anti-inflammatory effect of adiponectin on human gingival fibroblasts (HGFs) and mouse gingival fibroblasts (MGFs). In addition, we examined the physiological effect of adiponectin on cytodifferentiation of human PDL (HPDL) cells. The results showed that adiponectin suppressed proinflammatory cytokines induced by interleukin (IL)-1β stimulation, possibly via AdipoR1. Furthermore, adiponectin promoted the differentiation and mineralization of HPDL cells.

Material and methods

Reagents

Recombinant human and mouse IL-1β, adiponectin and normal rabbit IgG were obtained from R&D Systems (Minneapolis, MN, USA). Anti-adipoR1 IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Alpha Diagnostic Intl. Inc. (San Antonio, TX, USA).

Cells

Before participating in this study, all human subjects provided informed consent according to a protocol that was reviewed and approved by the Institutional Review Board of the Osaka University Graduate School of Dentistry. HGFs were obtained from biopsies of healthy gingiva taken from healthy volunteers, as previously described (18). HGFs were used for experiments at passages 4–10. MGFs were isolated from healthy gingival tissue of the first premolar teeth of BALB/c mice. When the cells that grew out from the explants reached confluence, they were separated by treatment with 0.53 mM EDTA containing 0.05% trypsin, collected by centrifugation and cultured on plastic culture dishes containing standard medium (standard medium is α-minimal essential medium containing 10% fetal calf serum) until they reached confluence. After 12 passages, the clonal MGF cell line was established using the limiting-dilution method. HPDL cells were isolated and maintained as described previously (19,20). For the induction of cytodifferentiation, HPDL cells were cultured in α-minimal essential medium (α-MEM) containing 10% fetal calf serum, 10 mM β-glycerophosphate and 50 μg/mL of ascorbic acid [(calcification-inducing medium (C-Med)]. C-Med was replaced every 3 d.

RT-PCR

Total RNA was isolated from HGFs, HPDL cells and MGFs using an RNA-Bee kit (TEL-TEST, Inc., Friendswood, TX, USA) according to the manufacturer's instructions. cDNA was synthesized and amplified using PCR, as described previously (18). Oligonucleotide PCR primers specific for adiponectin and AdipoRs were synthesized by Clontech (Palo Alto, CA, USA). The sequences of the primers are shown in Table 1. Hypoxanthine phosphoribosyltransferase (HPRT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as housekeeping genes.

Western blot analysis

HGFs, HPDL cells and MGFs were lysed in RIPA buffer [25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 1% sodium deoxycholate, 0.1% SDS, 10 mM Na_3VO_4 and 10 μg/mL each of aprotinin and leupeptin]. The proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with 10% bovine serum albumin for 1 h and subsequently with rabbit polyclonal anti-adiponectin Ig (Alpha Diagnostic International inc.) or goat polyclonal anti-AdipoR Ig (Santa Cruz Biotechnology) for 1 h at

Table 1. Primers used for RT-PCR

Gene	Sequence		
Human	Forward	5′-CGA GAT GTG ATG AAG GAG ATG GG-3′	304 bp
HPRT1	Reverse	5′-GCC TGA CCA AGG AAA GCA AG TC-3′	
Human	Forward	5′-CAA ACA GCC CCA AAG TCA AT-3′	288 bp
Adiponectin	Reverse	5′-TCT CAG GTG AGG TGG GAA AC-3′	
Human	Forward	5′-AAA CTG GCA ACA TCT GGA CC-3′	300 bp
AdipoRl	Reverse	5′-GCT GTG GGG AGC AGT AGA AG-3′	
Human	Forward	5′-ACA GGC AAC ATT TGG ACA CA-3′	267 bp
AdipoR2	Reverse	5′-CCA AGG AAC AAA ACT TCC CA-3′	
Mouse	Forward	5′-AGG TTG TCT CCT GCG ACT TC-3′	211 bp
GAPDH	Reverse	5′-CTT GCT CAG TGT CCT TGC TG-3′	
Mouse	Forward	5′-ATC TGA CGA CAC CAA AAG GG-3′	226 bp
Adiponectin	Reverse	5′-TCT CCA GGA GTG CCA TCT CT-3′	
Mouse	Forward	5′-TGC CCT CCT TTC GGG CTT GC-3′	529 bp
AdipoRl	Reverse	5′-GCC TTG ACA AAG CCC TCA GCG ATA G-3′	
Mouse	Forward	5′-TCT TCC TGT GCC TGG GGA TCT T-3′	254 bp
AdipoR2	Reverse	5′-CCC GAT ACT GAG GGG TGG CAA A-3′	

AdipoR1, adiponectin receptor 1; *AdipoR2*, adiponectin receptor 2; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *HPRT1*, hypoxanthine phosphoribosyltransferase-1.

room temperature and appropriate horseradish peroxidase-conjugated secondary antibody. Immune complexes were detected using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Waltham, MA, USA).

Real-time PCR

HGFs and MGFs were seeded in a six-well plate at a density of 3×10^5 cells and 1.2×10^6 cells/well, respectively. Cells were grown to confluence in standard medium. Following 18 h of preincubation in the presence or absence of adiponectin, cells were treated with or without 0.1 ng/mL of IL-1β, then total RNA was isolated and precipitated. cDNA was synthesized and mixed with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and gene-specific primers (Takara Bio, Shiga, Japan). Real-time PCR was performed using a 7300 Fast Real-Time PCR System (Applied Biosystems). The sequences of the primers are shown in Table 2. HPRT and GAPDH served as housekeeping genes.

Measurement of inflammatory cytokines in culture supernatants

HGFs and MGFs were seeded in a 12-well plate at a density of 1.8×10^5 and 7.2×10^5 cells, respectively, and grown to confluence in standard medium. Following 18 h of preincubation with or without adiponectin, cells were treated with or without 0.5 ng/mL of IL-1β. In some experiments, cells were pretreated for 1 h with anti-adiponectin Ig. At the end of the incubation periods, the supernatants were collected and the levels of IL-6 and IL-8 (HGFs only) protein were measured using ELISA kits (R&D Systems) according to the manufacturer's instructions.

RNA interference

Small interfering RNA (siRNA) was used to knock down the expression of mouse AdipoR1 and AdipoR2. The AdipoR1 and AdipoR2 siRNAs, and a negative-control siRNA (Silencer Select Negative Control #1 siRNA), were synthesized by Applied Biosystems. Silencer 1 Negative Control #1 siRNA was designed to have no significant sequence similarity to mouse, rat or human transcript sequences. MGFs were placed on a six-well culture dish. Twenty-four hours after incubation, MGFs, at 40–50% confluence, were transfected with siRNA AdipoR1, siRNA AdipoR2 or negative-control siRNA. The cells were transfected with 200 pmol of siRNA and negative-control siRNA using Lipofectamine 2000 (Invitrogen Corp, Carlsbad, CA, USA) according to the

Table 2. Primers used for real-time PCR

Gene	Sequence	
Human	Forward	5′-GGC AGT ATA ATC CAA AGA TGG TCA A-3′
HPRT1	Reverse	5′-GTC AAG GGC ATA TCC TAC AAC AAA C-3′
Human	Forward	5′-AAG CCA GAG CTG TGC AGA TGA GTA-3′
IL6	Reverse	5′-TGT CCT GCA GCC ACT GGT TC-3′
Human	Forward	5′-ACA CTG CGC CAA CAC AGA AAT TA-3′
IL8	Reverse	5′-TTT GCT TGA AGT TTC ACT GGC ATC-3′
Human	Forward	5′-GGA CCA TTC CCA CGT CTT CAC-3′
ALP	Reverse	5′-CCT TGT AGC CAG GCC CAT TG-3′
Human	Forward	5′-CAC TGG CGC TGC AAC AAG A-3′
RUNX2	Reverse	5′-CAT TCC GGA GCT CAG CAG AAT AA-3′
Mouse	Forward	5′-TGT GTC CGT CGT GGA TCT GA-3′
GAPDH	Reverse	5′-TTG CTG TTG AAG TCG CAG GAG-3′
Mouse	Forward	5′-CCA CTT CAC AAG TCG GAG GCT TA-3′
IL6	Reverse	5′-GCA AGT GCA TCA TCG TTG TTC ATA C-3′

ALP, alkaline phosphatase; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *HPRT1*, hypoxanthine phosphoribosyltransferase-1; *IL6*, interleukin-6; *IL8*, interleukin-8; *RUNX2*, runt-related transcription factor 2.

manufacturer's instructions. The cells were then analyzed using real-time PCR.

Determination of alkaline phosphatase activity, and staining with Alizarin Red

Alkaline phosphatase (ALP) activity was assessed according to the procedure described previously (20,21). Histochemical analysis of calcified nodules was performed using the Alizarin Red staining method (20,22). The density of calcified nodules in each well was calculated using the WinRoof software program (Mitani Corporation, Fukui, Japan).

Statistical analysis

The results were analyzed for statistical significance using the Student's *t*-test. Differences were considered significant at $p < 0.05$.

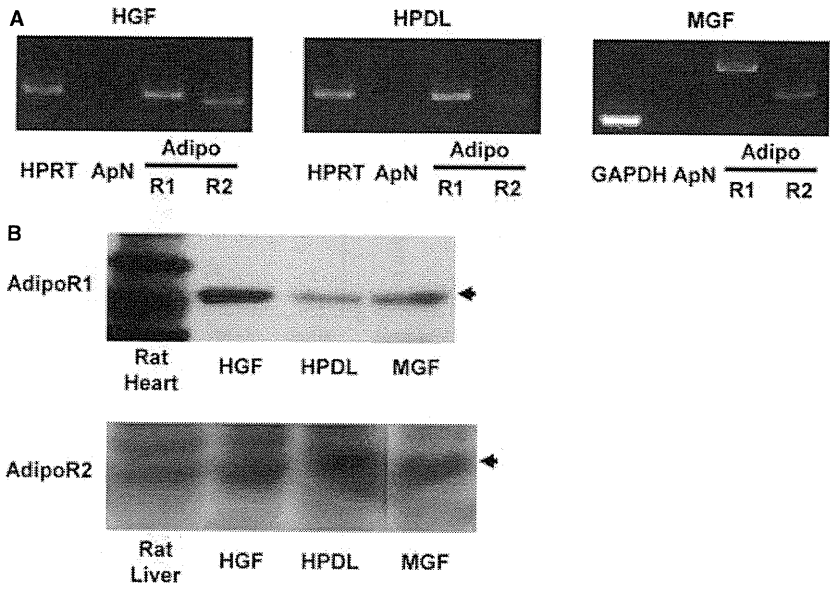

Fig. 1. Expression of adiponectin (ApN), adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2). (A) Expression of *ApN*, *AdipoR1* and *AdipoR2* genes in human gingival fibroblasts (HGFs), human periodontal ligament (HPDL) cells and mouse gingival fibroblasts (MGFs) was examined by RT-PCR. (B) Expression of AdipoR1 and AdipoR2 proteins in HGFs, HPDL cells and MGFs was detected using western blotting. The data represent one of three independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT, hypoxanthine phosphoribosyltransferase.

Results

Expression of AdipoR1 and AdipoR2, but not adiponectin, was detected in HGFs, HPDL cells and MGFs

To examine the expression of adiponectin and its receptors (AdipoR1 and AdipoR2) and mRNA and protein in HGFs, HPDL cells and MGFs, we performed RT-PCR amplification and western blotting. As shown in Fig. 1A and 1B, mRNA transcripts and protein for AdipoR1 and AdipoR2, but not for adiponectin, were detected in all cell types investigated.

Adiponectin reduced the expression of proinflammatory cytokines in IL-1β-stimulated HGFs

To the effect of adiponectin on the expression of proinflammatory cytokines in IL-1β-stimulated HGFs, HGFs were pretreated with adiponectin for 18 h before 2.5 h of stimulation with IL-1β, whereupon real-time PCR was performed. As shown in Fig. 2A, adiponectin significantly reduced the expression of *IL6* and *IL8* mRNAs. For assessment of IL-6 and IL-8 production, adiponectin-pretreated HGFs were stimulated with 0.5 ng/mL of IL-1β, harvested after 12 h and then assayed using ELISA. Adiponectin also reduced the production of IL-6 protein and IL-8 protein (Fig. 2B).

AdipoR1 knockdown abrogated the adiponectin-induced reduction of IL-6 expression in MGFs

To examine the effect of adiponectin on the expression of IL-6 in IL-1β-stimulated MGFs, MGFs were pretreated with adiponectin for 18 h before stimulation with IL-1β for 2.5 h, whereupon real-time PCR was performed. As shown in Fig. 3A, adiponectin significantly reduced the expression of *IL6* mRNA. To assess the production of IL-6 protein, adiponectin-pretreated MGF were stimulated with 0.5 ng/mL of IL-1β, harvested after 12 h and then assayed using ELISA. Adiponectin also reduced the production of IL-6 protein (Fig. 3B). To elucidate the effect of adiponectin via AdipoRs, siRNA was used to block the expression of *AdipoR1* and *AdipoR2* mRNAs. As shown in Fig. 4A and 4B, real-time PCR revealed that treatment with siRNA-AdipoR1 and siRNA-AdipoR2 significantly reduced the expression of AdipoR1 and AdipoR2 in MGFs compared with negative-control siRNA (Fig. 4A and 4B). Whereas IL-1β-induced expression of IL-6 in MGF treated with negative-control siRNA was significantly suppressed by adiponectin treatment, these suppressive effects were attenuated in MGFs treated with siRNA for AdipoR1 (Fig. 4C). By contrast, treatment with siRNA for AdipoR2 did not suppress IL-1β-induced expression of IL-6 by adiponectin (Fig. 4D). These results suggest that adiponectin reduces IL-6 expression in MGFs possibly via AdipoR1.

Anti-AdipoR1 Ig attenuated the adiponectin-induced reduction of IL-6 expression in HGF

To elucidate the effect of adiponectin via AdipoR1, HGFs were pretreated with control antibodies or with two types of anti-AdipoR1 polyclonal Igs and then stimulated with IL-1β in the presence or absence of adiponectin.

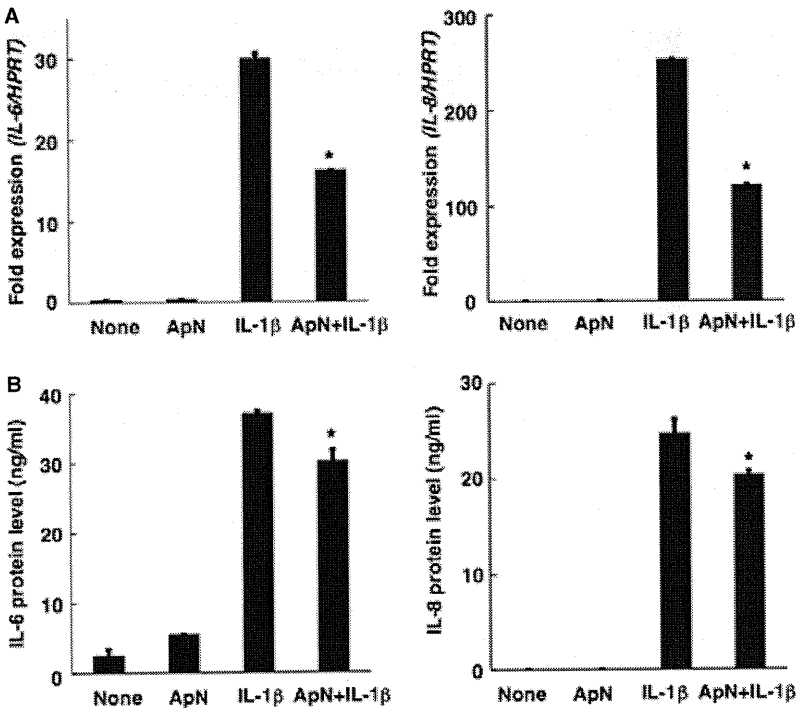

Fig. 2. Adiponectin reduced interleukin (IL)-1β-induced production of IL-6 and IL-8 in human gingival fibroblasts (HGFs). (A) Expression of *IL6* and *IL8* genes in HGFs was examined using real-time PCR. HGFs were pretreated with adiponectin (ApN; 5 μg/mL) for 18 h, stimulated with IL-1β (0.1 ng/mL) for 2.5 h and then total RNA was isolated. (B) The levels of IL-6 and IL-8 protein in HGF cultured condition medium were measured using ELISA. HGFs were pretreated with adiponectin (ApN; 10 μg/mL) for 18 h and stimulated for 12 h with IL-1β (0.5 ng/mL). Data are the mean ± standard deviation of triplicate determinations. $*p < 0.05$ compared with IL-1β-stimulated HGF without ApN pretreatment. HPRT, hypoxanthine phosphoribosyltransferase.

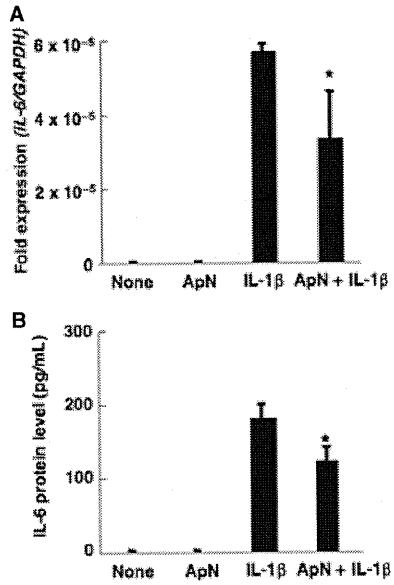

Fig. 3. Adiponectin reduced interleukin (IL)-1β-induced expression of IL-6 in mouse gingival fibroblasts (MGFs). (A) Expression of the *IL6* gene in MGFs was examined by real-time PCR. MGFs were pretreated with adiponectin (ApN; 20 μg/mL) for 18 h, stimulated with IL-1β (0.5 ng/mL) for 2.5 h and then total RNA was isolated. (B) The level of IL-6 protein in MGF cultured condition medium was measured by ELISA. MGFs were pretreated with adiponectin (ApN; 20 μg/mL) for 18 h and stimulated with IL-1β (0.5 ng/mL) for 12 h. Data are the mean ± standard deviation of triplicate determinations. $*p < 0.05$ compared with IL-1β-stimulated MGFs without pretreatment of adiponectin. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

After 12 h, IL-6 production in the culture supernatants was assayed using ELISA. As shown in Fig. 5, anti-AdipoR1 Igs significantly attenuated the suppression of IL-1β-induced IL-6 expression by adiponectin. These results suggest that adiponectin reduces IL-6 expression in the IL-1β-stimulated HGFs possibly via AdipoR1.

Adiponectin promoted the differentiation and mineralization of HPDL cells

Next, we examined whether or not adiponectin would promote the differentiation and mineralization of HPDL cells. As shown in Fig. 6A, ALP activity in HPDL cells was significantly enhanced in the presence of adiponectin. Real-time PCR revealed that adiponectin significantly enhanced the expression of *ALP* and runt-related transcription factor 2 (*Runx2*; an important transcription factor involved in osteoblastic differentiation and mineralization) (23) genes in HPDL cells cultured with C-Med in the presence of adiponectin (Fig. 6B and 6C) compared with C-Med only. Subsequently, mineralized nodule formation by HPDL cells on day 18 was investigated. As shown in Fig. 7A and 7B, adiponectin significantly increased the intensity of Alizarin Red staining. These results suggest that adiponectin promotes the differentiation and mineralization of HPDL cells.

Discussion

Adiponectin is an abundant serum protein, with concentrations in the order of 3–30 μg/mL (10). In this study, we demonstrated, for the first time, that physiological concentrations of adiponectin suppress IL-1β-induced IL-6 and IL-8 expression in HGFs, and IL-6 in MGFs, at mRNA and protein levels, possibly via AdipoR1 signaling. We also showed that adiponectin enhances the differentiation and mineralization of HPDL cells. Unfortunately, however, mouse IL-8 has not yet been identified. As mouse CXCL1 is known to be the functional homolog of human IL-8, we examined the expression CXCL1 in preliminary studies (data not shown). In these studies, we found that adiponectin significantly reduced the expression of *CXCL1* mRNA. However, the expression of CXCL1 protein was not reduced by adiponectin (data not shown).

Several previous studies have reported that adiponectin is the immunomodulatory cytokine for the function of monocytes, macrophages and

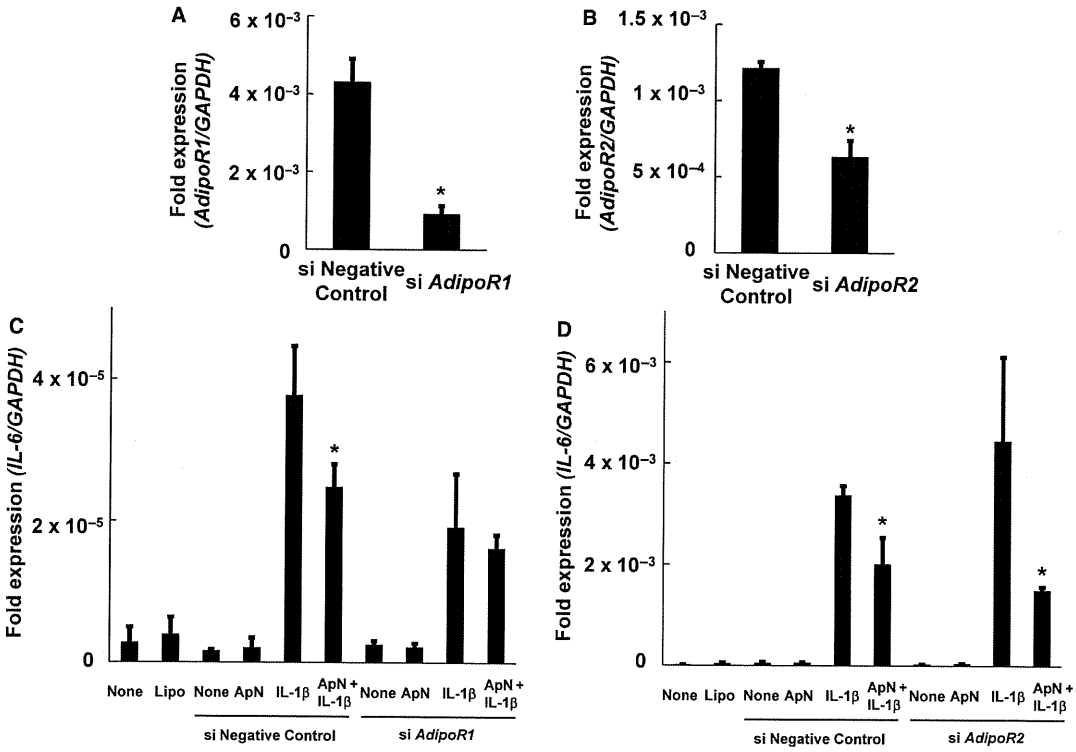

Fig. 4. Silencing adiponectin receptor 1 (AdipoR1) using small interfering (si)RNA attenuated the suppression of interleukin-6 (*IL6*) gene expression in mouse gingival fibroblasts (MGFs) stimulated with IL-1β. (A and B) MGFs transfected with AdipoR1, AdipoR2, or negative-control siRNAs were cultured for 24 h. *AdipoR1* and *AdipoR2* mRNAs were quantified using real-time PCR. These data represent one of three independent experiments. Data are the mean ± standard deviation of triplicate determinations (*$p < 0.05$). (C and D) Expression of the *IL6* gene in MGFs was examined using real-time PCR. MGFs transfected with AdipoR1, AdipoR2, or negative-control siRNAs were cultured for 24 h. The cells were seeded, pretreated with adiponectin (ApN; 20 μg/mL) for 18 h, stimulated with IL-1β (0.5 ng/mL) for 2 h and then total RNA was isolated. Data are the mean ± standard deviation of triplicate determinations. *$p < 0.05$ compared with IL-1β-stimulated MGFs without ApN pretreatment. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Lipo, lipofectamine 2000.

endothelial cells (10) and exhibits functional activity through binding to two AdipoRs. A recent systematic review concluded that there was a positive association between periodontal disease and obesity across diverse populations (3). The plasma adiponectin level in obese individuals was decreased compared with that in non-obese individuals (24). Saito *et al.* (11) previously reported that serum adiponectin levels in women with periodontitis were lower than in those with healthy gingiva, although this difference was not significant. Additionally, Yamaguchi *et al.* (14) revealed that the expression levels of AdipoRs in regions of periodontal disease were lower than in healthy gingival tissue. In mice with collagen-induced arthritis, adiponectin mitigated the severity of diseases (25). In addition, adiponectin suppressed the expression of inflammatory cytokines in stimulated rheumatoid arthritis synovial fibroblasts (25). We found that HGFs, HPDL cells and MGFs expressed AdipoR1 and AdipoR2, but not adiponectin (Fig. 1). Additionally, we showed that suppression of AdipoR1 expression by its siRNA and anti-AdipoR1 Ig abrogated the anti-inflammatory actions of the cells, suggesting that adiponectin promoted these responses through the action of AdipoR1, at least in part on gingival fibroblasts (Figs 4 and 5).

Recent studies have demonstrated that AdipoRs are also expressed on osteoblasts (15,16,26). In the present study we showed that AdipoR1 and AdipoR2 expressed on HPDL cells and adiponectin enhanced ALP activity, expression of *ALP* and *Runx2* genes, and mineralized nodule formation in HPDL cells (Figs 6 and 7). ALP is an enzyme marker of osteoblasts and participates in mineralization (27). Runx2 has been identified as an important transcription factor that is involved in bone formation and osteoblast differentiation (23). Thus, enhanced expression of *ALP* and *Runx2* genes, stimulated by adiponectin, were correlated with the mineralization of HPDL cells. In this study we did not directly examine the possible involvement of AdipoR1 in differentiation and mineralization of HPDL cells. However, a recent study reporting that adiponectin induced the differentiation and mineralization of osteoblastic MC3T3-E1 via AdipoR1 (26) suggests that the adiponectin–AdipoR1 pathway is also involved in the cytodifferentiation of HPDL cells. Although the functional roles of adiponectin in periodontal tissues have not been fully clarified, adiponectin should be involved in maintaining the homeostasis of periodontal tissue.

In this study, we demonstrated two functional aspects of adiponectin: the

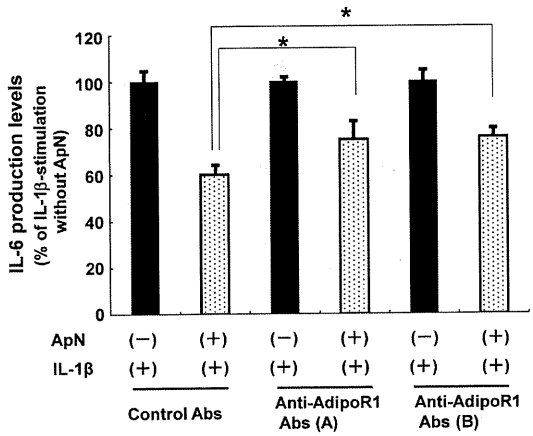

Fig. 5. Anti-adiponectin receptor 1 (AdipoR1) Igs attenuated the suppression of interleukin (IL)-6 production in human gingival fibroblasts (HGFs) stimulated with IL-1β. Cells were pretreated with control antibodies or with anti-AdipoR1 Igs for 1 h, incubated with adiponectin (ApN; 10 μg/mL) for 18 h and then stimulated with IL-1β (0.5 ng/mL) for 12 h. The levels of IL-6 protein in HGF cultured condition medium were measured using ELISA. Data are the mean ± standard deviation of triplicate determinations. Normalized data are shown as a percentage of the value in HGFs pretreated with the respective antibodies in the absence of ApN. $*p < 0.05$ compared with control antibody-pretreated HGFs in the presence of ApN.

first was an anti-inflammatory effect on gingival fibroblasts and the second was enhancement of cytodifferentiation in HPDL cells. Although we did not perform studies to clarify the signal pathway initiated following the activation of AdipoR1, accumulating evidence suggests that adiponectin increases the activities of sirtuin1 (SIRT1) and adenosine monophosphate-activated protein kinase (AMPK; 10). SIRT1 is an NAD^+-dependent deacetylase that interacts with and deacetylates p65 of nuclear factor-κB and subsequently inhibits the expression of inflammatory genes (28). Adiponectin has been shown to increase the levels of SIRT1 protein and to suppress lipopolysaccharide (LPS)-stimulated tumor necrosis factor-α production in Kupffer cells (29). AMPK is a heterotrimeric serine kinase responsive to a variety of cellular stimuli. In osteoblastic cells, AMPK is

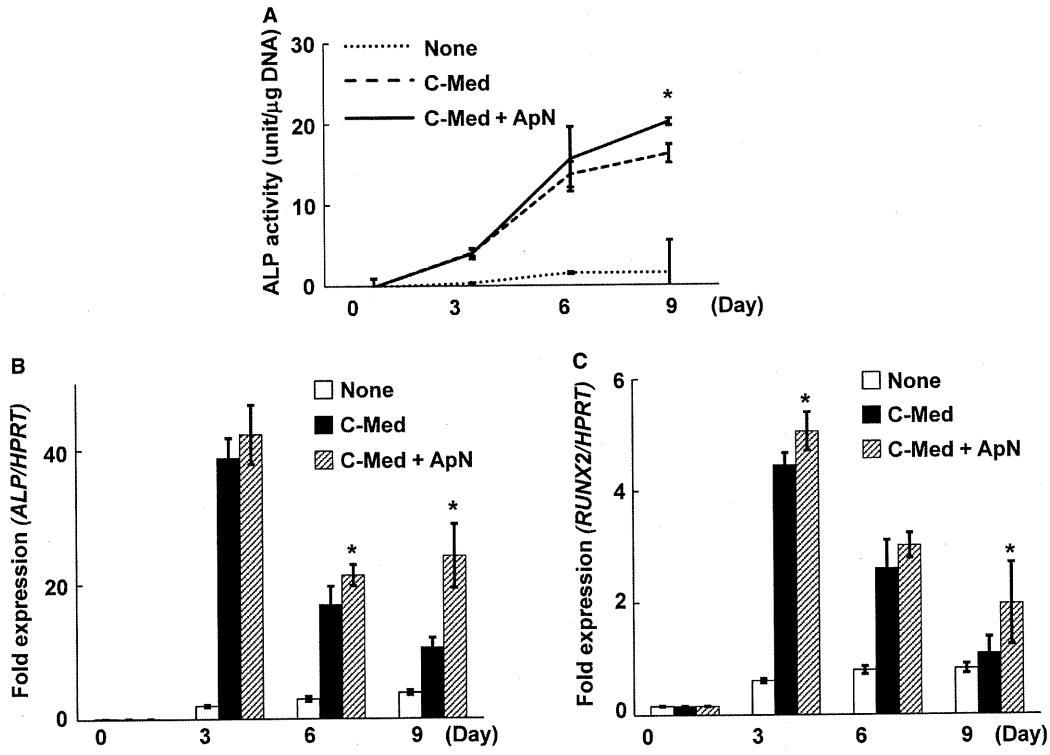

Fig. 6. Adiponectin promoted the differentiation of human periodontal ligament (HPDL) cells. (A) HPDL cells were cultured in calcification-induced medium (C-Med) in the presence or absence of adiponectin (ApN; 10 μg/mL) for the indicated periods of time, then alkaline phosphatase (ALP) activity during the cytodifferentiation of HPDL cells was measured. Data are the mean ± standard deviation of triplicate determinations. $*p < 0.05$ compared with HPDL cells cultured in C-Med in the absence of ApN. (B and C) Real-time PCR was performed to determine the expression of cytodifferentiation- and mineralization-related genes, such as *ALP* (B) and runt-related transcription factor 2 (*RUNX2*) (C). HPDL cells were cultured in C-Med in the presence or absence of adiponectin (ApN; 10 μg/mL) for the indicated periods of time. Data are the mean ± standard deviation of triplicate determinations. $*p < 0.05$ compared with HPDL cells cultured in C-Med in the absence of adiponectin. HPRT, hypoxanthine phosphoribosyltransferase.

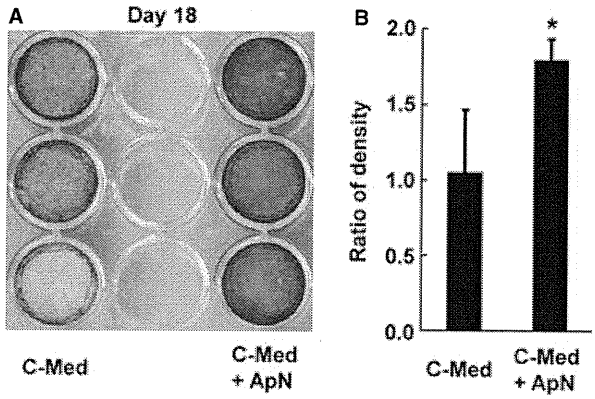

Fig. 7. Adiponectin promoted the mineralization of human periodontal ligament (HPDL) cells. (A) Mineralized nodule formation in HPDLs was detected by staining the cells with Alizarin Red after 18 d of culture in calcification-induced medium (C-Med) in the presence or absence of adiponectin (ApN; 10 μg/mL). (B) The relative expression value of Alizarin Red staining is shown in (A) and was quantified and normalized to Alizarin Red staining without C-Med. The data represent one of three independent experiments. $*p < 0.05$ compared with C-Med only.

stimulated via AdipoR1 and induces the production of bone morphogenetic protein-2, stimulating cells to differentiate into calcified (26,30). Further studies are necessary to clarify the involvement of the SIRT1 and AMPK signaling pathways in the action of adiponectin on both gingival fibroblasts and HPDL cells.

In summary, the results of the present study suggest that adiponectin may have a therapeutically beneficial effect on the control of anti-inflammatory responses and treatment of periodontal diseases. Topical application of recombinant adiponectin during periodontal surgery may improve wound healing and regeneration at the periodontal disease site. Further studies are still required to demonstrate this therapeutic effect.

Acknowledgements

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Periodontal disease in a patient with Prader-Willi syndrome: a case report

Manabu Yanagita, Hiroyuki Hirano, Mariko Kobashi, Takenori Nozaki, Satoru Yamada, Masahiro Kitamura and Shinya Murakami*

Abstract

Introduction: Prader-Willi syndrome is a complex genetic disease caused by lack of expression of paternally inherited genes on chromosome 15q11-q13. The prevalence of Prader-Willi syndrome is estimated to be one in 10,000 to 25,000. However, descriptions of the oral and dental phenotype are rare.

Case presentation: We describe the clinical presentation and periodontal findings in a 20-year-old Japanese man with previously diagnosed Prader-Willi syndrome. Clinical and radiographic findings confirmed the diagnosis of periodontitis. The most striking oral findings were anterior open bite, and crowding and attrition of the lower first molars. Periodontal treatment consisted of tooth-brushing instruction and scaling. Home care involved recommended use of adjunctive chlorhexidine gel for tooth brushing twice a week and chlorhexidine mouthwash twice daily. Gingival swelling improved, but further treatment will be required and our patient's oral hygiene remains poor. The present treatment of tooth-brushing instruction and scaling every three weeks therefore only represents a temporary solution.

Conclusions: Rather than being a direct result of genetic defects, periodontal diseases in Prader-Willi syndrome may largely result from a loss of cuspid guidance leading to traumatic occlusion, which in turn leads to the development of periodontal diseases and dental plaque because of poor oral hygiene. These could be avoided by early interventions to improve occlusion and regular follow-up to monitor oral hygiene. This report emphasizes the importance of long-term follow-up of oral health care by dental practitioners, especially pediatric dentists, to prevent periodontal disease and dental caries in patients with Prader-Willi syndrome, who appear to have problems maintaining their own oral health.

Introduction

Prader-Willi syndrome (PWS) is a complex multi-system genetic disorder that results from abnormalities in the critical region of chromosome 15q11.2-q13, including paternal interstitial deletion, maternal uniparental disomy and imprinting defects [1,2]. PWS is characterized by infantile hypotonia, poor suck, hyperphagia and subsequent obesity, hypogonadism, mental retardation and various learning disabilities. In addition, PWS is associated with a variety of musculoskeletal abnormalities including scoliosis, short hands and feet, facial dysmorphy, narrow hands with straight ulnar border, delayed bone age and joint hyperlaxity. Characteristics of this syndrome indicate hypothalamic dysfunction, and the incidence of the disorder is estimated at 1 in 10,000 to 25,000 individuals. The disorder can be divided into several stages [1,2]. The neonatal stage is mainly characterized by severe hypotonia and poor suck, which improves with age. Hyperphagia and subsequent obesity present in infancy, and small stature, developmental delay and behavioral problems (rigidity, stubbornness) are manifested during childhood. Adolescence is characterized by incomplete and delayed puberty, infertility, and deterioration of oppositional behavior (persistence of annoying behaviors, tantrums, and obsessive-compulsive behavior). Management of PWS, therefore, requires a multidisciplinary professional, parental and social approach to reduce morbidity and improve quality of life [3].

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Previously described oral and dental features of PWS include hypoplastic enamel [4,5], rampant caries [4,5], and dental erosion [6]. With respect to periodontal diseases, only one previous report has documented early-onset periodontitis in a 12-year-old girl with PWS [7]. We report a case of PWS in a 20-year-old man who presented to our facility with severe and localized periodontitis.

Case presentation

A 20-year-old Japanese man presented with his father to our periodontic clinic at Osaka University Dental Hospital, Japan, because of his poor oral condition. His chief complaint was severely painful gums, which led to crying. He was unable to provide detailed information about when and how this problem had started. His father told us that our patient had thought there was a gingival problem since childhood, had complained of gingival pain for the previous six months, and had been unable to brush his teeth for the last two weeks. Our patient's height was 153 cm and his weight was 62 kg. His medical history included a three-month hospital stay caused by low birth weight and cyanosis just after birth. A clinical diagnosis of PWS had been made on the basis of symptoms such as hypotonia, genital hypoplasia, acromicria (short hands and feet) and genetic testing.

An intra-oral examination revealed poor oral hygiene with heavy generalized plaque throughout the permanent dentition. His gingival tissues showed marginal redness, swelling, and food impaction (Figure 1). An apparent anterior open bite, increasing overjet, an anterior crowded arch and malpositioning of the teeth were noted. In addition, circular caries and attrition of the mandibular first molars were present. Pocket depths ranged from 4 mm to 8 mm. Mobility grade 2 was present in the mandibular left second premolar. We tried to measure the pocket depth and clinical attachment level at his first visit. Unfortunately, however, our patient did not allow the pocket measurement because of pain. Thus, we could not perform the conventional pocket measurement and examined only the mesial and buccal/labial pockets. Periapical radiographs disclosed localized vertical bone resorption (mesiolateral of maxillary right first molar and mesiolateral of mandibular left first molar) (Figure 2). Of particular note, the mesiopalatal pocket depth of the maxillary right first molar was 8 mm.

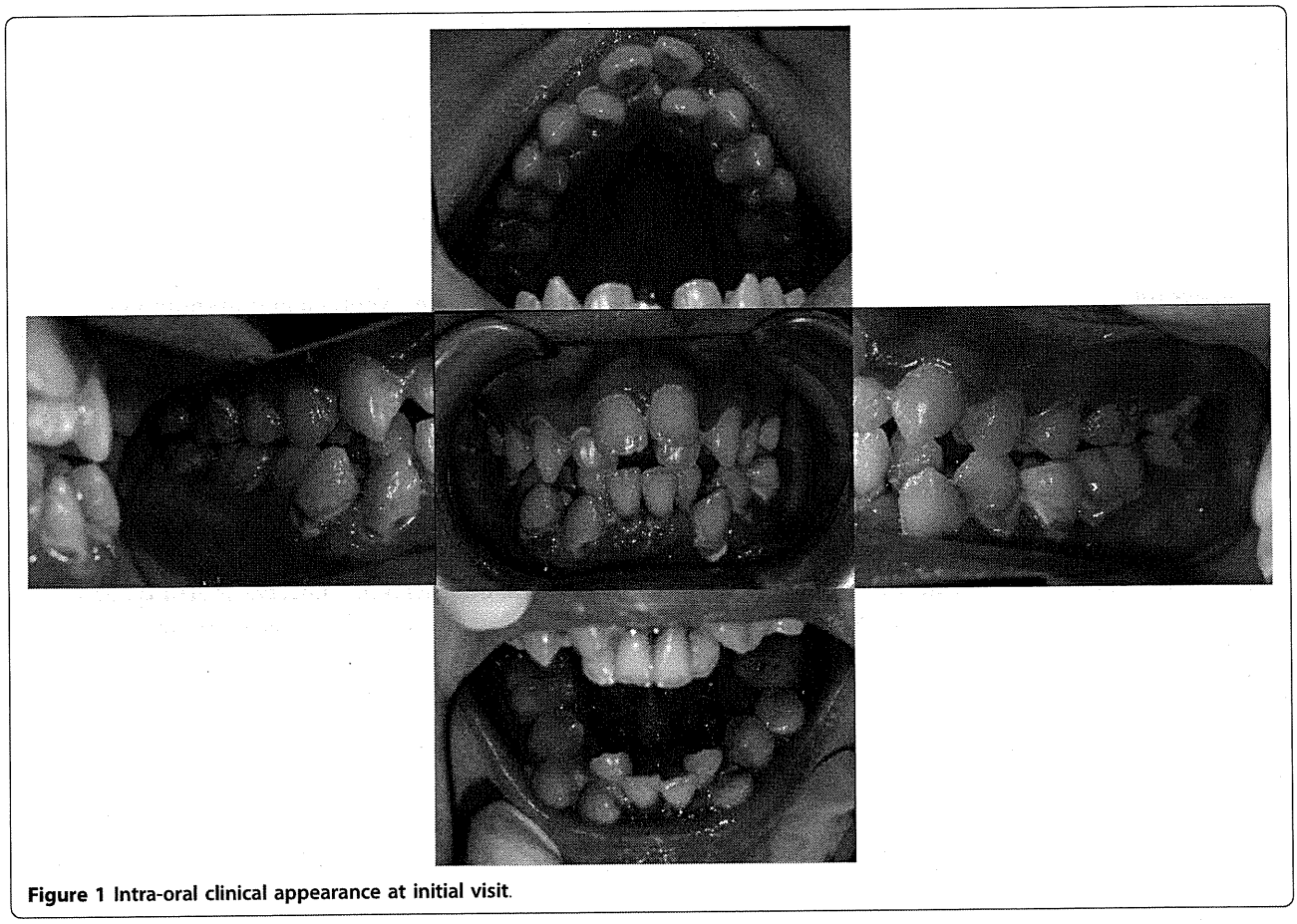

Figure 1 Intra-oral clinical appearance at initial visit.

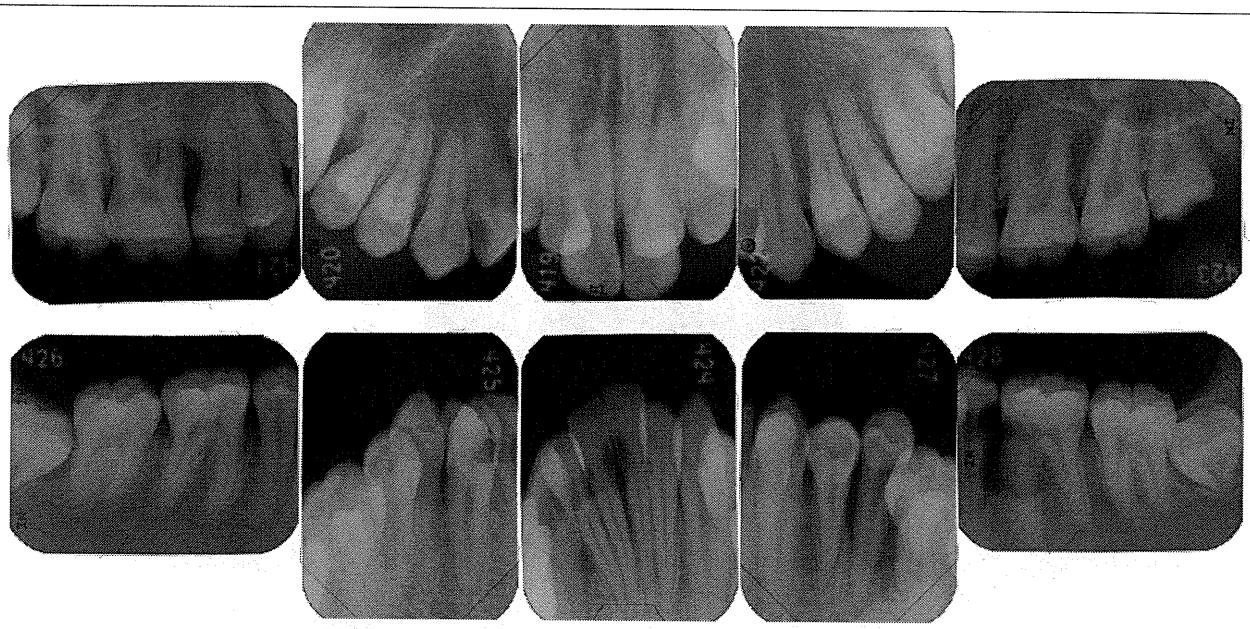

Figure 2 Baseline full-mouth periapical radiograph.

Based on pocket measurements and an X-ray examination, our patient was diagnosed with localized periodontitis. In addition, our patient exhibited several caries lesions from the mandibular right anterior teeth to the left molars. An orthopantomogram (Figure 3) showed full permanent teeth with unerupted lower third molars. There was no family history of periodontitis.

Advice on periodontal treatment was provided in the presence of one of our patient's parents. This included oral hygiene instructions on how to control plaque using a manual toothbrush (Sam Friend Supersoft #300; Sun Dental, Osaka, Japan), 0.2% w/w chlorhexidine mouth rinse (ConCool F; Weltech, Osaka, Japan) and 1% v/w chlorhexidine gel (ConCool Gelcoat F; Weltech). Professional scaling was also performed to remove supragingival plaque. Our patient kept his treatment appointments with his father every three weeks, but his plaque control was poor. His father told us that our patient was motivated to brush his teeth and did so happily, but sometimes he fell asleep without brushing because of daytime somnolence; a common occurrence in PWS [3]. He refused to allow his parents to help him brush his teeth. Furthermore, malpositioning of the teeth and difficulties with hand and wrist movements inhibited adequate plaque control. After three visits to the clinic our patient had become accustomed to the dental treatment, and subgingival scaling was performed using an ultrasonic scaler. However, active treatments such as root planing and periodontal surgery were not employed because of poor plaque control. Both toothbrushing instruction (TBI) and subgingival scaling were performed every three weeks. Although some gingival inflammation remained, his gingival swelling and redness were reduced by six months after his first visit (Figure 4).

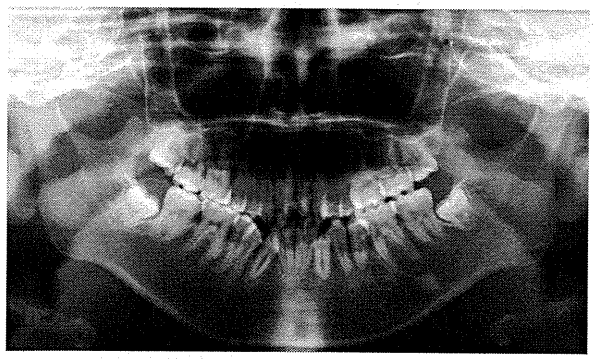

Figure 3 Panoramic radiograph.

Discussion

Most reports on the dental findings in PWS have focused on the presence of rampant caries, tooth wear, delayed tooth eruption, and hypoplastic enamel [4-6]. However, little is known about periodontal diseases in PWS. To the best of our knowledge, the only report in the English literature concerned a case of early-onset periodontitis in a 12-year-old girl with PWS [7]. The present report demonstrates the presence of moderate-to-severe periodontal destruction in a patient with PWS.

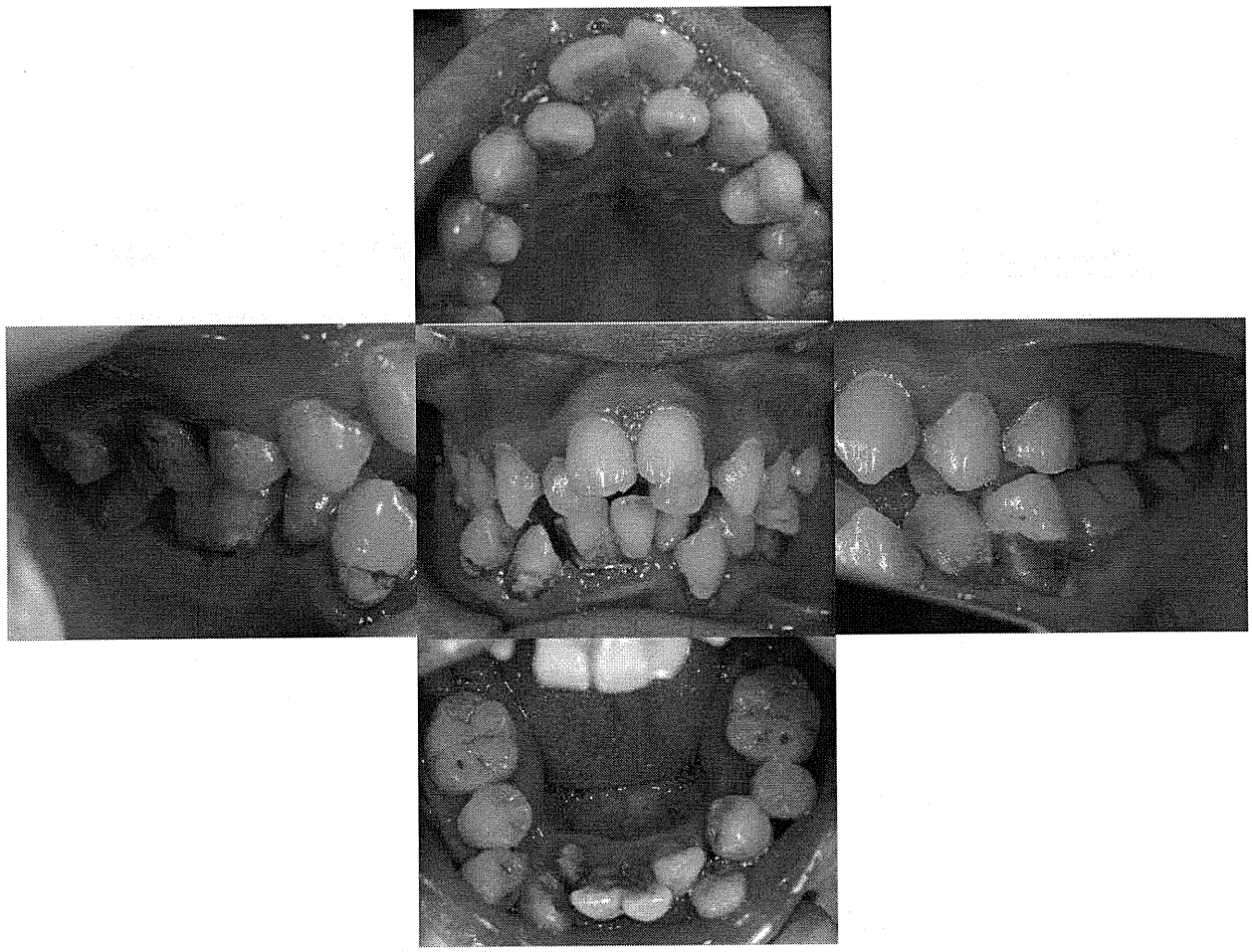

Figure 4 Clinical image at six months showing some improvement of gingival swelling.

Several researchers have reported severe periodontal diseases in patients with systemic genetic disorders such as Marfan syndrome (MFS), Ehlers-Danlos syndrome (EDS) and Down's syndrome (DS), which are accompanied by connective tissue disorders characterized by joint hypermobility and scoliosis. MFS is caused by mutations in the fibrillin-1 gene on chromosome 15q21.1, leading to abnormalities in the connective tissue matrix [8]. Straub *et al.* reported that inflammatory periodontal breakdown in a patient with MFS was caused by abnormalities in the periodontal connective tissue rather than being attributable to the primary biochemical changes of MFS [9]. EDS comprises a heterogeneous group of inherited connective tissue disorders characterized by joint laxity, chronic joint pain, skin hyperextensibility, tissue fragility and scoliosis [10]. EDS can be divided into 11 phenotypes, and periodontal diseases have been reported in EDS types I and VIII. The pathogeneses of these subtypes of EDS are unknown. The periodontal disease in EDS type I is localized, whereas EDS type VIII is characterized by ligneous periodontitis or persistent hyperplastic gingivitis [11]. DS is a chromosomal disorder that results from an extra copy of chromosome 21 (trisomy 21). Patients with DS have a variety of associated medical conditions, including joint hypermobility and ligamentous laxity [12]. The immune alternations in DS, including impaired neutrophil chemotactic function, are responsible for the defensive mechanisms in periodontal disease, which are frequently experienced by subjects with DS [13,14]. As in MFS, EDS and DS, musculoskeletal abnormalities such as scoliosis, joint hyperlaxity, delayed bone age and osteoporosis, are also clinical features of PWS [3]. Although a number of imprinted genes have been mapped to the PWS region at 15q11.2-q13, there is no case normal paternal copy of 15q11-q13 with inheritance consistent with a single mutated gene, suggesting that PWS is a multigenic syndrome [15]. Furthermore, there is no gene related to the connective tissue matrix in the PWS region. The periodontal breakdown