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# Adiponectin regulates functions of gingival fibroblasts and periodontal ligament cells

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*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 AAG 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 $3 \times 10^5$ cells and $1.2 \times 10^6$ 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 $1.8 \times 10^5$ and $7.2 \times 10^5$ 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 $p < 0.05$.

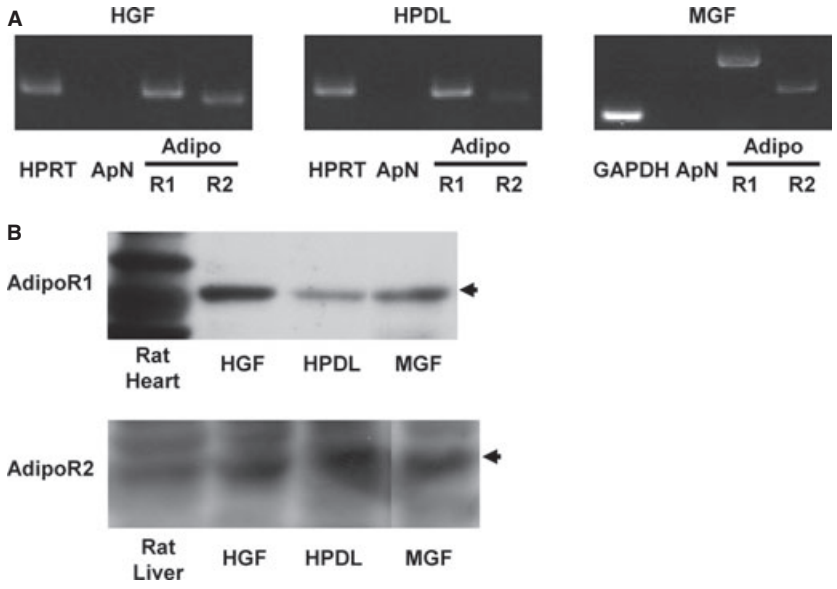


*Fig. 1.* Expression of adiponectin (ApN), adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2). (A) Expression of *ApN*, *AdipoR1* and *AdipoR2* genes in human gingival fibroblasts (HGFs), human periodontal ligament (HPDL) cells and mouse gingival fibroblasts (MGFs) was examined by 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.

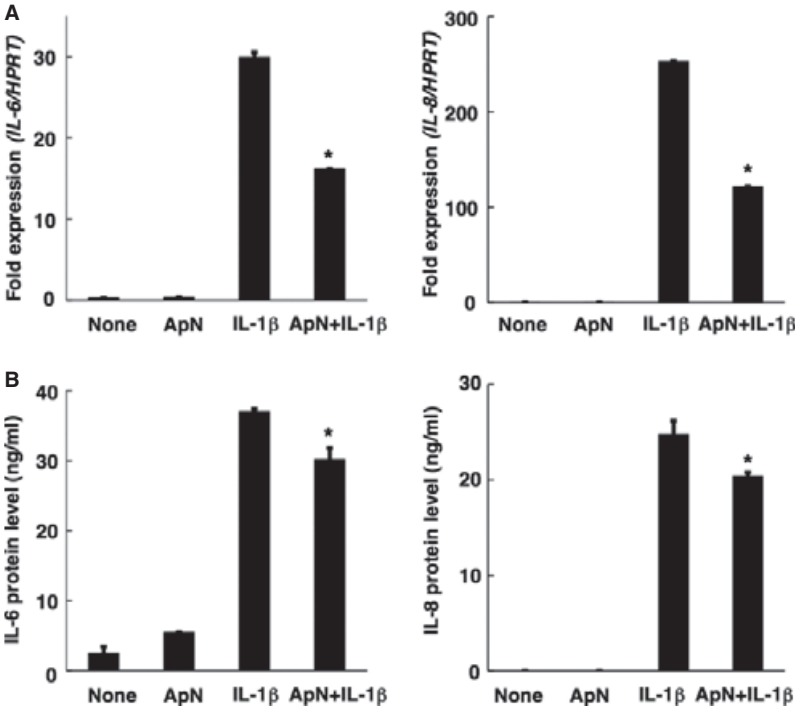


*Fig. 2.* Adiponectin reduced interleukin (IL)-1β-induced production of IL-6 and IL-8 in human gingival fibroblasts (HGFs). (A) Expression of *IL6* and *IL8* genes in HGFs was examined using real-time PCR. HGFs were pretreated with adiponectin (ApN; 5 μg/mL) for 18 h, stimulated with IL-1β (0.1 ng/mL) 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.

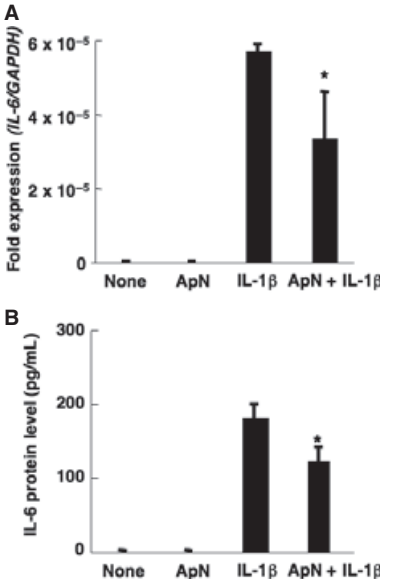


*Fig. 3.* Adiponectin reduced interleukin (IL)-1β-induced expression of IL-6 in mouse gingival fibroblasts (MGFs). (A) Expression of the *IL6* gene in MGFs was examined by real-time PCR. MGFs were pretreated with adiponectin (ApN; 20 μg/mL) for 18 h, stimulated with IL-1β (0.5 ng/mL) for 2.5 h and then 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

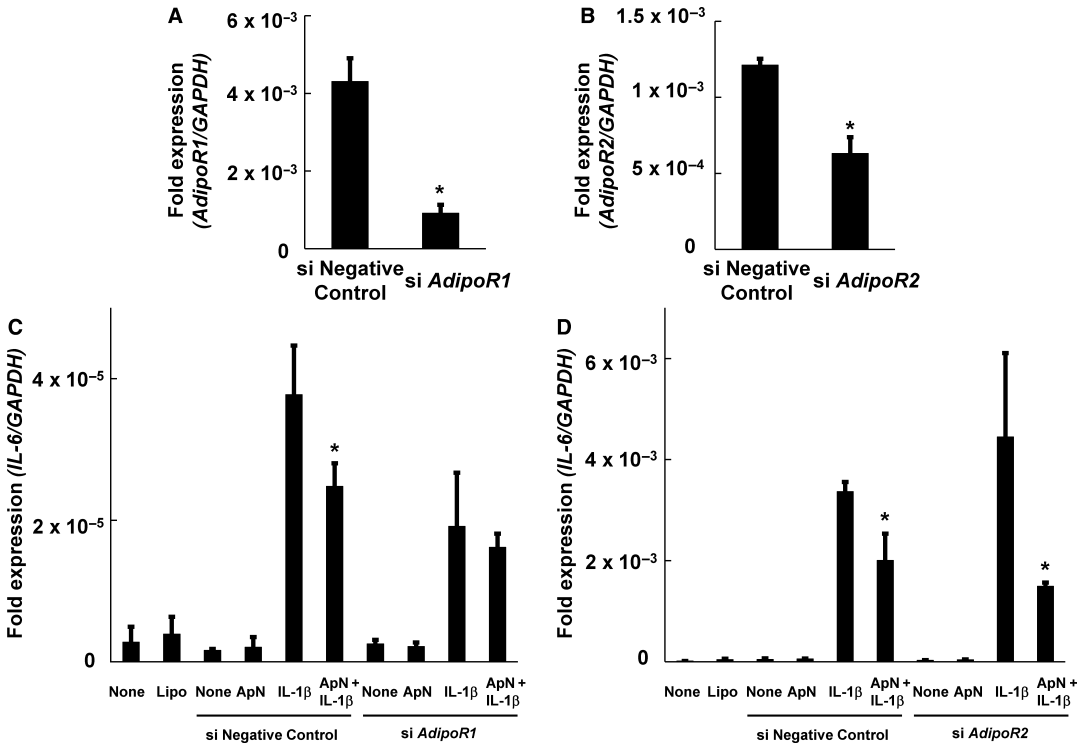


*Fig. 4.* Silencing adiponectin receptor 1 (AdipoR1) using small interfering (si)RNA attenuated the suppression of interleukin-6 (*IL6*) gene expression in mouse gingival fibroblasts (MGFs) stimulated with IL-1β. (A and B) MGFs transfected with AdipoR1, AdipoR2, or negative-control siRNAs were cultured 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

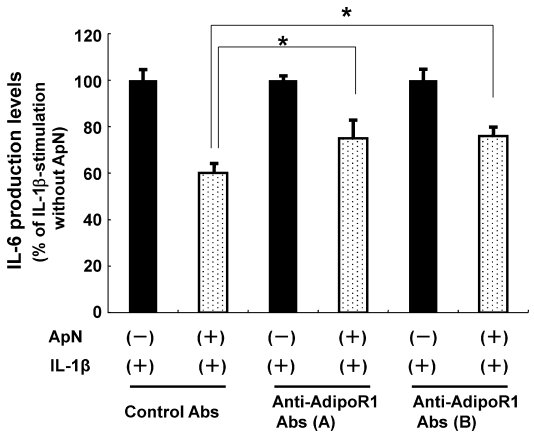


*Fig. 5.* Anti-adiponectin receptor 1 (AdipoR1) Igs attenuated the suppression of interleukin (IL)-6 production in human gingival fibroblasts (HGFs) stimulated with IL-1β. Cells were pretreated with control antibodies or with anti-AdipoR1 Igs for 1 h, incubated with adiponectin (ApN; 10 μg/mL) for 18 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

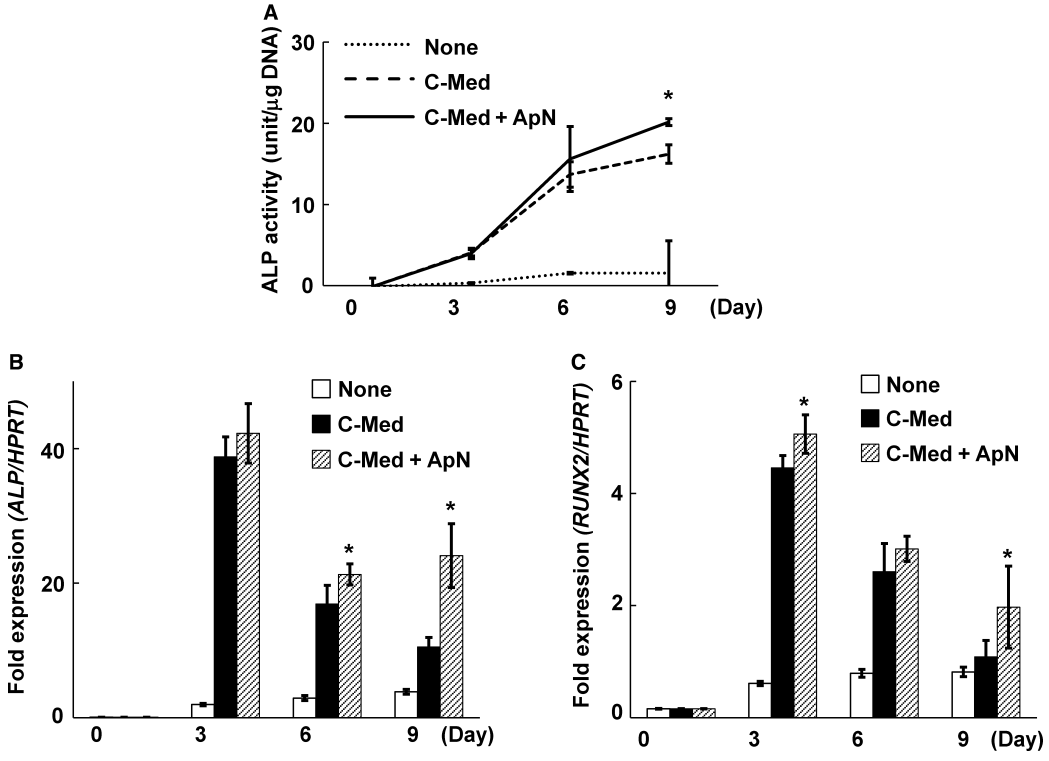


*Fig. 6.* Adiponectin promoted the differentiation of human periodontal ligament (HPDL) cells. (A) HPDL cells were cultured in calcification-induced medium (C-Med) in the presence or absence of adiponectin (ApN; 10 μg/mL) for the indicated periods of time, then alkaline phosphatase (ALP) activity during 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.

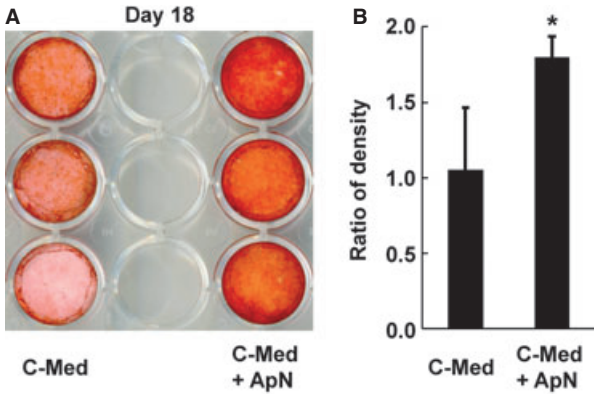


*Fig. 7.* Adiponectin promoted the mineralization of human periodontal ligament (HPDL) cells. (A) Mineralized nodule formation in HPDLs was detected by staining the cells with Alizarin Red after 18 d of culture in calcification-induced medium (C-Med) in the presence or absence of adiponectin (ApN; 10 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.

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## References

1. Garcia RI, Henshaw MM, Krall EA. Relationship between periodontal disease and systemic health. *Periodontol 2000* 2001;**25:**21–36.
2. Saito T, Shimazaki Y, Sakamoto M. Obesity and periodontitis. *N Engl J Med* 1998;**339:**482–483.
3. Chaffee BW, Weston SJ. Association between chronic periodontal disease and obesity: a systematic review and meta-analysis. *J Periodontol* 2010;**81:**1708–1724.
4. Kopelman PG. Obesity as a medical problem. *Nature* 2000;**404:**635–643.
5. Funahashi T, Nakamura T, Shimomura I *et al.* Role of adipocytokines on the pathogenesis of atherosclerosis in visceral obesity. *Intern Med* 1999;**38:**202–206.
6. Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF. A novel serum protein similar to C1q, produced exclusively in adipocytes. *J Biol Chem* 1995;**270:**26746–26749.
7. Yamauchi T, Kamon J, Ito Y *et al.* Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* 2003;**423:**762–769.
8. Ouchi N, Kihara S, Arita Y *et al.* Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponecti. *Circulation* 1999;**100:**2473–2476.
9. Yu JG, Javorschi S, Hevener AL *et al.* The effect of thiazolidinediones on plasma adiponectin levels in normal, obese, and type 2 diabetic subjects. *Diabetes* 2002;**51:**2968–2974.
10. Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. *Nat Rev Immunol* 2011;**11:**85–97.
11. Saito T, Yamaguchi N, Shimazaki Y *et al.* Serum levels of resistin and adiponectin in women with periodontitis: the Hisayama study. *J Dent Res* 2008;**87:**319–322.
12. Furugen R, Hayashida H, Yamaguchi N *et al.* The relationship between periodontal condition and serum levels of resistin and adiponectin in elderly Japanese. *J Periodontal Res* 2008;**43:**556–562.
13. Kardeşler L, Buduneli N, Cetinkalp S, Kinane DF. Adipokines and inflammatory mediators after initial periodontal treatment in patients with type 2 diabetes and chronic periodontitis. *J Periodontol* 2010;**81:**24–33.
14. Yamaguchi N, Hamachi T, Kamio N *et al.* Expression levels of adiponectin receptors and periodontitis. *J Periodontal Res* 2010;**45:**296–300.
15. Berner HS, Lyngstadaas SP, Spahr A *et al.* Adiponectin and its receptors are expressed in bone-forming cells. *Bone* 2004;**35:**842–849.
16. Oshima K, Nampei A, Matsuda M *et al.* Adiponectin increases bone mass by suppressing osteoclast and activating osteoblast. *Biochem Biophys Res Commun* 2005;**331:**520–526.
17. Seo BM, Miura M, Gronthos S *et al.* Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* 2004;**364:**149–155.
18. Murakami S, Hashikawa T, Saho T *et al.* Adenosine regulates the IL-1 beta-induced cellular functions of human gingival fibroblasts. *Int Immunol* 2001;**13:**1533–1540.
19. Somerman MJ, Archer SY, Imm GR, Foster RA. A comparative study of human periodontal ligament cells and gingival fibroblasts in vitro. *J Dent Res* 1988;**67:**66–70.
20. Fujihara C, Yamada S, Ozaki N *et al.* Role of mechanical stress-induced glutamate signaling-associated molecules in cytodifferentiation of periodontal ligament cells. *J Biol Chem* 2010;**285:**28286–28297.
21. Bessey OA, Lowly OH, Brock MJ. A method for the rapid determination of alkaline phosphates with five cubic millimeters of serum. *J Biol Chem* 1946;**164:**321–329.
22. Dahl LK. A simple and sensitive histochemical method for calcium. *Proc Soc Exp Biol Med* 1952;**80:**474–479.
23. Yang S, Wei D, Wang D, Phimphilai M, Krebsbach PH, Franceschi RT. In vitro and in vivo synergistic interactions between the Runx2/Cbfa1 transcription factor and bone morphogenetic protein-2 in stimulating osteoblast differentiation. *J Bone Miner Res* 2003;**18:**705–715.
24. Arita Y, Kihara S, Ouchi N *et al.* Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* 1999;**257:**79–83.
25. Lee SW, Kim JH, Park MC, Park YB, Lee SK. Adiponectin mitigates the severity of arthritis in mice with collagen-induced

arthritis. *Scand J Rheumatol* 2008;**37:**260–268.
26. Kanazawa I, Yamaguchi T, Yano S, Yamauchi M, Yamamoto M, Sugimoto T. Adiponectin and AMP kinase activator stimulate proliferation, differentiation, and mineralization of osteoblastic MC3T3-E1 cells. *BMC Cell Biol* 2007;**8:**51.
27. Majeska RJ, Wuthier RE. Studies on matrix vesicles isolated from chick epiphyseal cartilage. Association of pyrophosphatase and ATPase activities with alkaline phosphatase. *Biochim Biophys Acta* 1975;**391:**51–60.
28. Yang SR, Wright J, Bauter M, Seweryniak K, Kode A, Rahman I. Sirtuin regulates cigarette smoke-induced proinflammatory mediator release via RelA/p65 NF-kappaB in macrophages in vitro and in rat lungs in vivo: implications for chronic inflammation and aging. *Am J Physiol Lung Cell Mol Physiol* 2007;**292:**L567–L576.
29. Shen Z, Ajmo JM, Rogers CQ *et al*. Role of SIRT1 in regulation of LPS- or two ethanol metabolites-induced TNF-alpha production in cultured macrophage cell lines. *Am J Physiol Gastrointest Liver Physiol* 2009;**296:**G1047–G1053.
30. Huang CY, Lee CY, Chen MY, Tsai HC, Hsu HC, Tang CH. Adiponectin increases BMP-2 expression in osteoblasts via AdipoR receptor signaling pathway. *J Cell Physiol* 2010;**224:**475–483.