Journal of
PERIODONTAL RESEARCH

J Periodont Res 2010; 45: 296–300

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2009.01222.x

Short communication

Expression levels of adiponectin receptors and periodontitis

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Yamaguchi N, Hamachi T, Kamio N, Akifusa S, Masuda K, Nakamura Y, Nonaka K, Maeda K, Hanazawa S, Yamashita Y. Expression levels of adiponectin receptors and periodontitis. J Periodont Res 2010; 45: 296–300. © 2010 John Wiley & Sons A/S

Background and Objective: We recently showed that adiponectin, an adipocyte-derived cytokine, may function as a negative regulator of the Toll-like receptor signaling pathway and of osteoclast formation in periodontal disease. In this study, we investigated whether the expression levels of adiponectin receptors (AdipoR1 and AdipoR2) are related to the presence of periodontitis.

Material and Methods: We initially examined, using RT-PCR, the expression of the *AdipoR1* and *AdipoR2* genes at the mRNA level in several oral tissues of C57BL mice. Next, we investigated (using real-time PCR assays) whether inflammatory cytokines, such as tumor necrosis factor-α, could affect the expression levels of these genes in human gingival fibroblasts. Lastly, we compared the expression levels of these receptor proteins in gingival tissues between two healthy subjects and five patients with severe periodontal disease using western blotting analysis.

Results: The AdipoR1 and AdipoR2 receptors were ubiquitously expressed in the oral tissues of mice. We observed that treatment with tumor necrosis factor-α could significantly reduce the expression levels of both *AdipoR1* and *AdipoR2* genes in human gingival fibroblasts. Moreover, we found that the expression of both receptors was lower in periodontal tissues from patients with severe periodontitis than in patients with healthy periodontal tissues.

Conclusion: These observations suggest that adiponectin may not function efficiently in sites of periodontal disease because of a decrease in the number of its receptors, and this probable dysfunction may play a role in worsening periodontitis in patients.

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Key words: adiponectin receptor; fibroblasts; periodontitis; tumor necrosis factor-α

Accepted for publication February 27, 2009

Previous epidemiologic studies have suggested that periodontal disease is closely associated with obesity and glucose tolerance (1,2). Adipokines such as adiponectin, resistin and leptin, which are secreted by adipocytes, can affect inflammation and insulin sensitivity (3,4). Many studies have recognized that periodontitis is more prevalent in patients with diabetes and worsens with the progression of the disease (5,6). Adiponectin is abundantly present in the plasma of nondiabetic individuals (1.9–17 μg/mL) (7), but its mRNA expression and circulating levels are reduced in obese subjects and in those with type 2 diabetes (8). Our recent epidemiologic study showed that the serum levels of adiponectin in middle-aged Japanese women with severe periodontitis were slightly lower than in those with a healthy gingiva, although the difference was not significant (9). Therefore, it was of interest to us to explore the presence of periodontitis with respect to adiponectin.

Full-length adiponectin is known to exist in three forms – trimers, hexamers and high-molecular-weight multimers –

in the serum (10). Moreover, adiponectin can be cleaved to liberate an active fragment containing the C-terminal globular adiponectin domain (11). Two adiponectin receptors have been discovered: AdipoR1, which is abundantly expressed in skeletal muscle; and AdipoR2, which is predominantly expressed in the liver (12). AdipoR1 and AdipoR2 are predicted to contain 7-pass transmembrane domains; however, they are thought to be structurally and functionally distinct from G-protein-coupled receptors (12). AdipoR1 and AdipoR2 serve as receptors for globular adiponectin and full-length adiponectin, and mediate increased 5′-AMP-activated protein kinase (AMPK) (13) and peroxisome proliferator-activated receptor-α ligand activities (14). Obesity decreases not only plasma adiponectin levels but also AdipoR1 and AdipoR2 expression, hence causing adiponectin resistance, which leads to insulin resistance (15).

Ouchi *et al*. (16) previously reported that adiponectin suppressed tumor necrosis factor (TNF-α)-mediated inflammatory responses in human aortic endothelial cells. Recently, they showed that adiponectin could promote endothelial cell migration and stimulate the differentiation of these cells into capillary-like structures. Moreover, they indicated that adiponectin promoted angiogenesis via activation of AMPK and phosphatidylinositol 3-kinase Akt-dependent pathways in endothelial cells (17).

Based on the results of these studies, we recently showed that adiponectin may negatively regulate the Toll-like receptor signaling pathway and osteoclast formation via AMPK signaling in periodontal disease (18–20). In this study, we investigated whether the expression levels of adiponectin receptors (AdipoR1 and AdipoR2) are related to the presence of periodontitis.

Material and methods

Oral tissue specimens

All mouse oral tissues were obtained from 15-week-old male C57BL mice. All experimental procedures were conducted in accordance with the animal experimental guidelines of Kyushu University. The animals underwent general anesthesia with pentobarbital sodium.

A total of five patients with chronic periodontitis and two periodontally healthy individuals referred to the Department of Periodontology at the Dental Hospital, Kyushu University, were selected for this study (see Table 1). No subjects had any significant medical history, systemic diseases or disorders that might affect the outcome of periodontal therapy. We excluded smokers, obese subjects and/or patients with type 2 diabetes from participating in this study. All patients with chronic periodontitis (one man and four women, 50–60 years of age) exhibited severe periodontal destruction, having a probing pocket depth of > 5 mm in multiple sites. The periodontally healthy controls (two women, 56 and 72 years of age) had healthy gingiva, no radiographic evidence of bone loss and a probing pocket depth of < 3 mm. Normal gingival tissues were obtained from the sites of the third molars once they had been extracted. The gingival tissues of periodontitis patients were obtained during periodontal surgery, after completion of initial therapy, including motivation, oral hygiene instruction, scaling and root planing (see Table 1). The tissue samples obtained were immediately soaked in nitrogen and frozen at −80°C until examined. The Ethics Committee of Kyushu University Faculty of Dental Science approved this study, and informed consent was obtained from all patients before inclusion in the study.

Table 1. List of subjects and periodontal status of obtained gingival tissues

Subject	Age	Gender	BOP/Pus	PPD	Mobility
N1	72	F	−/−	2	M0
N2	56	F	−/−	2	M0
P1	56	F	+/−	7	M2
P2	60	M	+/+	7	M2
P3	50	F	+/−	6	M2
P4	50	F	+/+	7	M2
P5	51	F	+/+	8	M2

BOP, bleeding on probing; PPD, probing pocket depth (mm).

Reagents

Porphyromonas gingivalis lipopolysaccharide (Pg-LPS) was extracted from lyophilized cells of *P. gingivalis* strain 381 using the hot phenol-water procedure, treated with nuclease and washed extensively in pyrogen-free water by ultracentrifugation. This preparation was purified by chromatography on Sephadex G-200 (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) previously equilibrated with 10 mM Tris–HCl (pH 8.0) containing 0.2 M NaCl, 0.25% (w/v) deoxycholate, 1 mM EDTA and 0.02% (w/v) sodium azide (21). Lipopolysaccharide from *Escherichia coli* O111:B4 was purchased from Sigma-Aldrich Corp. (St Louis, MO, USA). Human recombinant TNF-α (specific activity, 2×10^7 U/mg) was obtained from PeproTech EC (London, UK).

Cell culture

Human gingival fibroblasts were prepared from explants of human normal gingival tissues. The explants were cultured in 30-mm-diameter Falcon plastic dishes with Dulbecco's modified Eagle's minimal essential medium (Sigma-Aldrich Corp.) containing 10% fetal bovine serum (Thermo Trace Ltd, Melbourne, Vic., Australia), 2 mM L-glutamine and 50 μg/mL of gentamicin under an atmosphere of 5% CO_2 at 37°C. After a confluent monolayer of the migrating cells had formed, the cells were passaged by trypsinization and then again grown to confluence in 90-mm-diameter plastic dishes. After the fifth passage, the cells were typical homogeneous fibroblasts with a spindle shape. We used the gingival fibroblasts between passages 6 and 10. The murine macrophage-like cell line RAW264 (RCB0535; RIKEN Cell Bank, Ibaragi, Japan) was maintained in RPMI-1640 (Sigma-Aldrich Corp.) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 50 μg/mL of gentamicin.

RT-PCR

Total RNA from mouse oral tissues or RAW264 cells was isolated by using the TRIzol reagent (Invitrogen Corp.,

Carlsbad, CA, USA). The RNA samples (5 μg) were reverse transcribed to cDNA by use of ready-to-go you-prime first-strand beads (GE Healthcare Bio-Sciences Corp.), and the cDNAs for AdipoR1, AdipoR2 and β-actin were amplified by PCR. PCR primers were partly designed from recently published sequences (12) and were as follows: AdipoR1 forward primer, 5′-ACGTTGGAGAGTCATC CCGTAT-3′ and AdipoR1 reverse primer, 5′-CTCTGTGTGGATGCG GAAGAT-3′ (product size: 130 bp); AdipoR2 forward primer, 5′-TCCCAGGAAGATGAAGGGTTTA T-3′ and AdipoR2 reverse primer, 5′-TTCCATTCGTTCCATAGCATGA-3′ (product size: 60 bp); β-actin forward primer, 5′-ATGGATGACGATATCG CT-3′, and β-actin reverse primer, 5′-ATGAGGTAGTCTGTCAGGT-3′ (product size: 588 bp). The resulting amplification products were electrophoresed on a 12.5% acrylamide gel and stained with ethidium bromide. The bands were visualized by illumination with ultraviolet light.

Real-time quantitative PCR

Human gingival fibroblasts were treated with human recombinant TNF-α (50 ng/mL) or Pg-LPS (100 ng/mL) and were then incubated for 0, 0.5, 1 or 4 h. Total RNA from the cells was isolated using an RNeasy plus mini kit (QIAGEN K.K., Tokyo, Japan). The RNA samples (5 μg) were reverse transcribed to cDNA by use of ready-to-go you-prime first-strand beads (GE Healthcare Bio-Sciences Corp.). TaqMan® β-actin control reagents (Applied Biosystems, Foster City, CA, USA) were used as an internal control. Human AdipoR1 and AdipoR2 mRNA levels were determined by conducting quantitative real-time PCR assays. Amplification and detection were performed using an ABI PRISM 7700 Sequence Detector (Perkin-Elmer Japan Co. Ltd, Applied Biosystems Division, Chiba, Japan), operated according to the following cycle profile: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. PCR primers were designed from recently published sequences (12) and were as follows: human AdipoR1 forward primer, 5′-TTCTTCCTCAT GGCTGTGATGT-3′, and human AdipoR1 reverse primer, 5′-AAGA AGCGCTCAGGAATTCG-3′; human AdipoR1 TaqMan probe, 5′-TCAC TGGAGCTGGCCTTTATGCTGC-3′; human AdipoR2 forward primer, 5′-ATAGGGCAGATAGGCTGGTT GA-3′, and human AdipoR2 reverse primer, 5′-GGATCCGGGCAGCAT-ACA-3′; human AdipoR2 TaqMan probe, 5′-CTGATGGCCAGCCTC-TACATCACAGGA-3′. Quantification of mRNA was performed by determining the threshold cycle, as described previously (19). Human AdipoR1 and AdipoR2 mRNA levels were normalized to those of the housekeeping gene, β-actin.

Western blotting

Human gingival tissues were homogenized in the T-PER® tissue protein extraction reagents (Pierce, Rockford, IL, USA). The prepared lysates (20 μg of protein) were resolved on 4–12% gradient gels and then transferred electrophoretically to nitrocellulose membranes. Immunoblotting was performed as described previously (20). The membranes were first exposed to primary antibodies and then to secondary antibodies conjugated with horseradish peroxidase. The primary antibodies used were anti-AdipoR1, anti-AdipoR2 (Alpha Diagnostic International, San Antonio, TX, USA) and anti-actin (MP Biomedicals Inc., Aurora, OH, USA).

Statistical analysis

For the real-time quantitative PCR assay, comparisons between groups were performed using Tukey's multiple range tests.

Results and Discussion

In the present study, we examined the expression levels of AdipoR1 and AdipoR2 in tissues of the oral cavity to explore the potential role of adiponectin in oral tissues. Both AdipoR1 and AdipoR2 receptors were ubiquitously expressed in mouse oral tissues as well as in RAW264 cells (Fig. 1). Both genes were also expressed in human gingival fibroblasts (data not shown). These findings showed that adiponectin could function effectively in relation to certain oral diseases.

In inflamed gingiva, gingival fibroblasts may be stimulated with various cytokines derived from host cells. TNF-α is a component of the early induction phase of the inflammatory cascade (22). The concentration used and incubation time of this cytokine were based on the study published by Kobayashi *et al*. (23). Our own titration of human recombinant TNF-α (50 ng/mL) in our preliminary experiments was more effective for inhibiting the expression of adiponectin receptors. Treatment with this concentration of TNF-α for 0.5–1 h reduced the expression level of the *AdipoR1* gene in human gingival fibroblasts ($p < 0.01$), as determined by conducting our real-time PCR assay using TaqMan probes (Fig. 2A). The expression of the AdipoR1 receptor reached basal levels after 4 h of exposure to TNF-α (Fig. 2A). By contrast, treatment with TNF-α for 0.5 h decreased the level of expression of the *AdipoR2* gene in human gingival fibroblasts ($p < 0.05$), reaching near-basal levels after 1 h of exposure to TNF-α (Fig. 2A). TNF-α can alter the production of many adipokines, which is relevant to the systemic effects of TNF-α on insulin sensitivity. TNF-α may induce systemic insulin resistance and dyslipidaemia by suppressing the

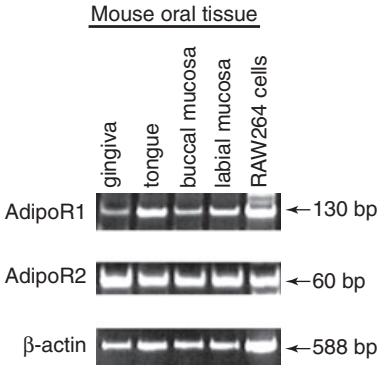

Fig. 1. RT-PCR analysis of mouse adiponectin receptors (AdipoR1 and AdipoR2) and β-actin in mouse oral tissues and RAW264 cells.

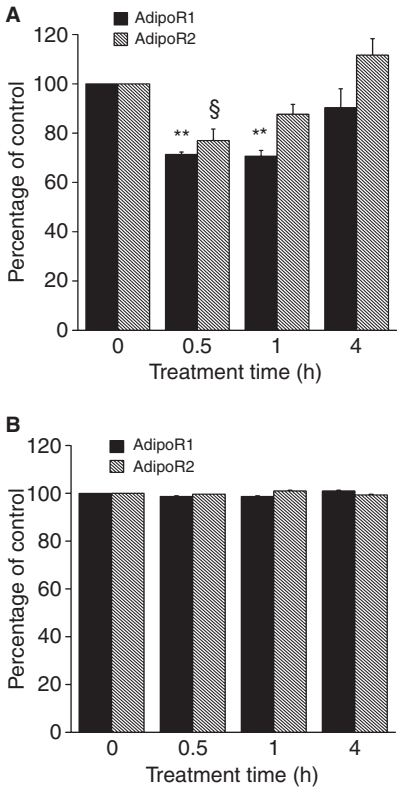

Fig. 2. Effect of tumor necrosis factor-α (TNF-α) (A) or *Porphyromonas gingivalis* lipopolysaccharide (Pg-LPS) (B) on the expression of adiponectin receptor genes (AdipoR1 and AdipoR2) in human gingival fibroblasts. The cells were treated with human recombinant TNF-α (50 ng/mL) or Pg-LPS (100 ng/mL), and were then incubated for 0, 0.5, 1 or 4 h. The mRNA levels of human AdipoR1 (closed bars) or AdipoR2 (cross-hatched bars) are presented relative to the untreated control. Results are means ± standard errors of three independent experiments. $^{**}p < 0.01$ vs. basal AdipoR1 gene expression level. $^{\S}p < 0.05$ vs. basal AdipoR2 gene expression level.

production of adiponectin (24). Circulating levels of adiponectin are inversely correlated with plasma levels of TNF-α (25). The mechanism by which this is mediated is likely to involve the suppression of peroxisome proliferator-activated receptor-γ (26), CCAAT/enhancer binding protein β (27) and/or JNK activation (28,29). Tumor necrosis factor-α receptor 1 (TNFR1) and TNFR2 are ubiquitously expressed transmembrane glycoproteins that undergo trimerization upon ligand binding. Results from numerous reports suggest that the signals transduced by TNFR1 mediate the majority of the effects of TNF-α on adipose tissue function. Several investigations identified that TNFR1-induced signals are required and sufficient to impair insulin action (30–32). Further studies are needed to clarify the mechanism of TNFR1-induced signaling to adiponectin receptors in human gingival fibroblasts.

Treatment with Pg-LPS for 0.5–4 h did not affect the expression levels of adiponectin receptors in human gingival fibroblasts (Fig. 2B). Treatment with LPS from *E. coli* O111:B4 gave the same results in human gingival fibroblasts as did treatment with Pg-LPS (data not shown). Toll-like receptor 4 is stimulated by LPS, and this stimulation results in the activation of both I kappa B kinase β/nuclear factor-κB and JNK/activating protein-1 signaling, culminating in the expression and secretion of pro-inflammatory cytokines/chemokines, including interleukin-1β, TNF-α, monocyte chemoattractant protein, etc. (33). However, the mechanism of Toll-like receptor 4-induced signaling to adiponectin receptors in human gingival fibroblasts also remains incompletely understood.

Moreover, our immunoblot analysis showed that the expression of both receptors was markedly decreased in periodontal tissues from patients with severe periodontitis compared with that in tissues from healthy subjects (Fig. 3). We revealed, for the first time, that periodontal diseases might be associated with reduced expression of adiponectin receptors. In this study, we excluded obese subjects and/or patients with type 2 diabetes, in whom the adiponectin level was reduced, to examine in detail the relationship between the expression levels of adiponectin receptors and periodontal disease. The decrease in AdipoR1 and AdipoR2 mRNA leads to a decrease in adiponectin binding and in the effects of adiponectin, which is termed adiponectin resistance. Tsuchida *et al.* (34) showed that insulin negatively regulates the expression levels of adiponectin receptors and adiponectin sensitivity. They also demonstrated that down-regulation of adiponectin receptors by insulin may be mediated via the phosphoinositide 3-kinase/Foxo1-dependent pathway.

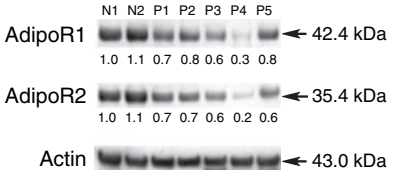

Fig. 3. Comparison of the expression levels of adiponectin receptors in human gingival tissues between periodontal healthy subjects and patients with severe periodontitis. Human gingival tissues from two periodontally healthy subjects were obtained when the impacted third molars were extracted. Human gingival tissues from five patients with periodontitis were taken during the flap operation, frozen in liquid nitrogen and stored at −80°C until examined. Protein extraction was carried out using the T-PER® tissue protein extraction reagents, and the proteins from each sample were electrophoresed on 4–12% gradient gels. Gels were blotted onto nitrocellulose membranes, and fluorographs were developed after incubation (overnight at 4°C) with anti-AdipoR1, anti-AdipoR2 or anti-actin. Relative band intensities were determined by using NIH image version 1.62 software. The values below the bands are the mean fold increases in the expression levels in gingival tissues compared with those of healthy subjects.

Our recent epidemiologic studies showed that the serum resistin level was associated with periodontitis in middle-aged Japanese women. By contrast, serum levels of adiponectin in women with periodontitis were slightly lower than in those with healthy gingiva, but the difference was not significant (9). Moreover, in elderly Japanese people, we obtained similar results, showing that increased serum resistin levels were significantly associated with periodontitis (35). However, the actual role of these adipokines in periodontitis has not yet been demonstrated.

Lee *et al.* (36) recently investigated the effect of adiponectin on rheumatoid arthritis by using rheumatoid arthritis synovial fibroblasts. Adiponectin significantly mitigated the severity of the arthritis and reduced the expression of TNF-α, interleukin-1β, and MMP-3 in stimulated rheumatoid

arthritis synovial fibroblasts. Their data suggest that adiponectin may play an anti-inflammatory role in the pathophysiology of rheumatoid arthritis.

In conclusion, our present observations suggest that adiponectin may not function efficiently in the periodontal disease site because of the decrease in the expression levels of adiponectin receptors. Subsequent probable adiponectin resistance may play a role in worsening periodontitis in patients.

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