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The effect of *Porphyromonas gingivalis* infection on cytokine levels in type 2 diabetic mice

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Background and Objective: Several studies have shown that diabetes mellitus increases the severity of periodontitis. Conversely, periodontitis has been shown to have an impact on diabetes, although the underlying mechanisms of this are unclear. The aim of this study was to compare the inflammatory response to *Porphyromonas gingivalis* infection in normal and diabetic mice.

Material and Methods: Porphyromonas gingivalis were inoculated adjacent to the periosteum, at a point on the midline of the skull located between the ears, in C57BL/6 (normal) and KKAy (diabetic) mice. After induction, the levels of tumor necrosis factor-α, interleukin-6 and adiponectin in the mice were measured using real-time polymerase chain reaction and enzyme-linked immunosorbent assay.

Results: The KKAy mice showed significant increases in blood glucose, serum tumor necrosis factor-α and interleukin-6 levels after inoculation with *Porphyromonas gingivalis*, and a significant decrease in adiponectin to 35.7%. Similar results were observed at the mRNA level in liver and visceral adipose tissue.

Conclusion: These observations suggest that tumor necrosis factor-α, interleukin-6 and adiponectin are an integral part of the link between diabetes mellitus and *Porphyromonas gingivalis* infection.

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Periodontitis is the most common oral infection in humans and is the main cause of tooth loss in adults (1,2). Diabetes mellitus is thought to be an important risk factor for periodontitis, and major periodontal tissue destruction has been reported in patients with diabetes mellitus (3,4). Moreover, impaired neutrophil function is thought to be related to severe periodontitis in patients with diabetes mellitus, and high concentrations of serum glucose can also lead to indirect damage through the formation of advanced-glycation end-products (5,6). Degenerative microvascular alterations are known complications of diabetes that occur in various tissues, including the periodontium (7,8). Thickening of the capillary basement membrane results in impaired oxygen exchange and altered transfer of metabolic products between the intracellular and extracellular compartments, ultimately affecting the host response and tissue repair.

Recent studies have suggested that a bidirectional inter-relationship exists between diabetes and periodontitis (9,10). Furthermore, current therapies for periodontal infection contribute to positive glycemic control and reduce the potential for complications related to diabetes mellitus (11,12). Periodontitis

is characterized by a progressive gingival inflammatory response to periodontopathic bacteria such as *Porphyromonas gingivalis* (*P. gingivalis*), and increasing evidence suggests that local inflammation might trigger a systemic host response, predisposing subjects with periodontitis to diabetes mellitus (13,14). Little evidence, however, is available regarding the underlying mechanisms. The aim of this study was to compare the inflammatory response to *P. gingivalis* infection with metabolic markers in normal and diabetic mice.

Material and methods

Mice

Male KKAy and C57BL/6 mice (6 wk of age) were obtained from CLEA Japan Inc. (Tokyo, Japan). KKAy mice, which are generated from C57BL/6J mice, are obese and develop diabetes spontaneously. Type 2 diabetes mellitus is characterized by an impaired insulin sensitivity and resultant dysregulation of glucose and lipid metabolism. KKAy mice exhibit morbid obesity and metabolic abnormalities, such as hyperglycemia, glucose intolerance and hyperinsulinemia, and are known to serve as an excellent model of type 2 diabetes mellitus (15). Fifty mice of each strain were used. The mice were housed individually at a constant temperature (23 ± 2°C) and 55 ± 5% relative humidity under a 12-h light/dark cycle (lights on at 07:00) and had free access to food and water. We observed the general condition of the mice and measured their body weight daily. Peripheral venous blood was taken from the tail of each mouse, and the fasting plasma glucose level was measured using a glucose measuring device (Bayer Medical Inc., Tuttlingen, Germany). All procedures were approved by the Animal Experimentation Committee at Nihon University School of Dentistry, Tokyo, Japan.

Inoculation with *P. gingivalis*

P. gingivalis FDI 381 was used in all experiments. Bacteria were maintained on Brucella HK agar (Kyokuto Pharmaceutical, Tokyo, Japan) supplemented with 10% horse blood under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) at 37°C. Growth in liquid media was monitored at an optical density of 550 nm. *P. gingivalis* cells were collected during logarithmic growth, washed three times with sterile phosphate-buffered saline and suspended in sterile phosphate-buffered saline. The mice were then immunized and subsequently challenged with *P. gingivalis*, as described previously (16). For immunization, the bacteria were fixed with 1% paraformaldehyde for 4 h prior to injection. An inoculum of 2.5×10^8 cells in sterile phosphate-buffered saline was injected subcutaneously into the dorsal dermis of the animals once weekly for three consec-

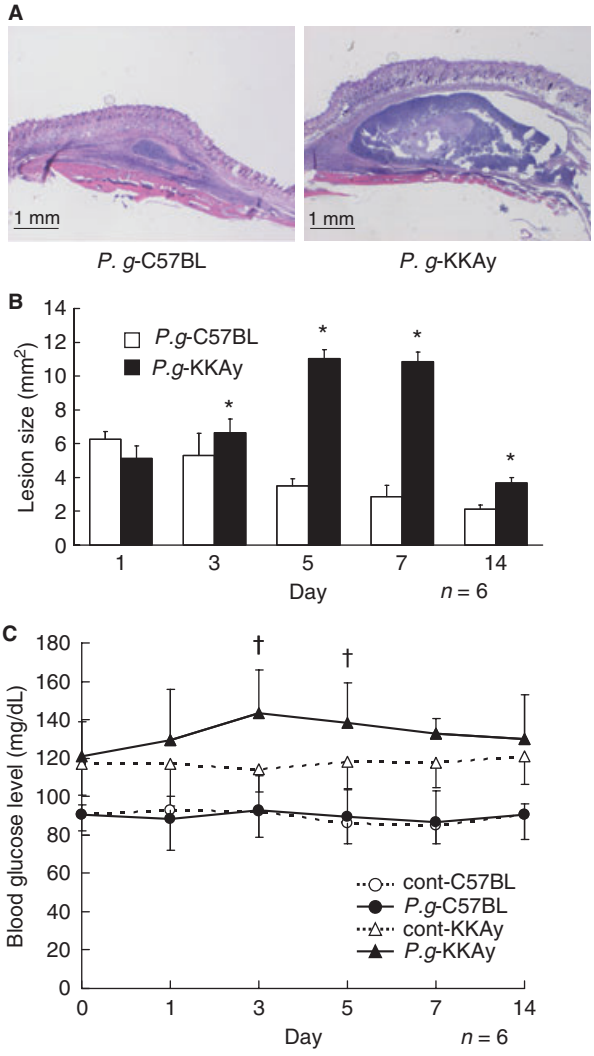

Fig. 1. Effect of inoculation with *P. gingivalis* on the body of C57BL and KKAy mice. (A) Four weeks after the first immunization, *P. gingivalis* was inoculated adjacent to the periosteum at a point on the midline of the skull between the ears. Calvaria with intact soft tissue were prepared for histologic sectioning. Five-micrometer-thick sections were stained with hematoxylin and eosin. (B) The sizes of the lesions were measured, and their areas were determined using a computerized image-analysis system. The bars represent the mean abscess size in mm^2. *$p < 0.05$ vs. *P.g*-C57BL. (C) Peripheral blood was taken from the tail vein and the fasting blood glucose level was measured at each time-point. †$p < 0.05$ vs. *P.g*-KKAy 0 d. cont-C57BL, control C57BL mice; cont-KKAy, control KKAy mice; *P.g*-C57BL, C57BL mice inoculated with *P. gingivalis*; *P.g*-KKAy, KKAy mice inoculated with *P. gingivalis*.

utive weeks. Four weeks after the first inoculation, live *P. gingivalis* (2.5×10^8 cells) were inoculated adjacent to the periosteum at a point on the midline of the skull located between the ears (16,17).

mRNA expression

For RNA isolation, liver and visceral adipose tissue was immediately fixed in an RNA-stabilization reagent (RNA-later™; Qiagen, Valencia, CA, USA). The samples were then homogenized, and total RNA was extracted using an RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. The mRNA expression of several cytokines was assessed by real-time polymerase chain reaction (PCR) using primers and probe sets purchased from Applied Biosystems (Foster City, CA, USA). Briefly, first-strand cDNA synthesis was achieved using 3 μg of total RNA from each sample and a first-strand synthesis kit (Amersham Biosciences, Sunnyvale, CA, USA). Real-time PCR was performed on an ABI PRISM 7700 Sequence Detector (Applied Biosystems) with the following cycling parameters: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and primer extension at 60°C for 1 min. The results were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each experiment was performed in duplicate.

Enzyme-linked immunosorbent assay

The serum levels of tumor necrosis factor-α, interleukin-6 and adiponectin in the mice were measured using enzyme-linked immunosorbent assay kits (tumor necrosis factor-α and interleukin-6: BioSource International, Inc., Camarillo CA, USA; adiponectin: Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan), according to the manufacturers' instructions.

Histological evaluation

Calvaria with intact soft tissue were prepared for histologic sectioning by fixation overnight in 4% paraformaldehyde at 4°C. After fixation, the specimens were decalcified and embedded in paraffin. Five-micrometer-thick sections were then cut and stained with hematoxylin and eosin. All abscess lesions were subsequently measured, and their areas (expressed as mm^2) were determined using a computerized image-analysis system (NIH IMAGE; NIH, Bethesda, MD, USA).

Statistical analysis

Each data point represents at least six mice. All data were presented as means ± standard deviation. The data for the KKAy and C57BL/6 mice were compared using a Mann–Whitney *U*-test, with a *p*-value of < 0.05 considered to be statistically significant. Comparisons between day 0 and each subsequent data point were made using Wilcoxon's signed rank sum test, with a *p*-value of < 0.05 considered to be statistically significant.

Results

A more pronounced inflammatory infiltrate was present in the KKAy mice compared with the C57BL/6 mice day 5 after inoculation with *P. gingivalis* (Fig. 1A). The maximum lesion sizes in each group are shown in Fig. 1B. When bacterial cells were injected into C57BL/6 mice, abscess lesions were observed on day 1, which gradually receded. By contrast, the abscesses reached their maximum size on days 5 and 7 in the KKAy mice,

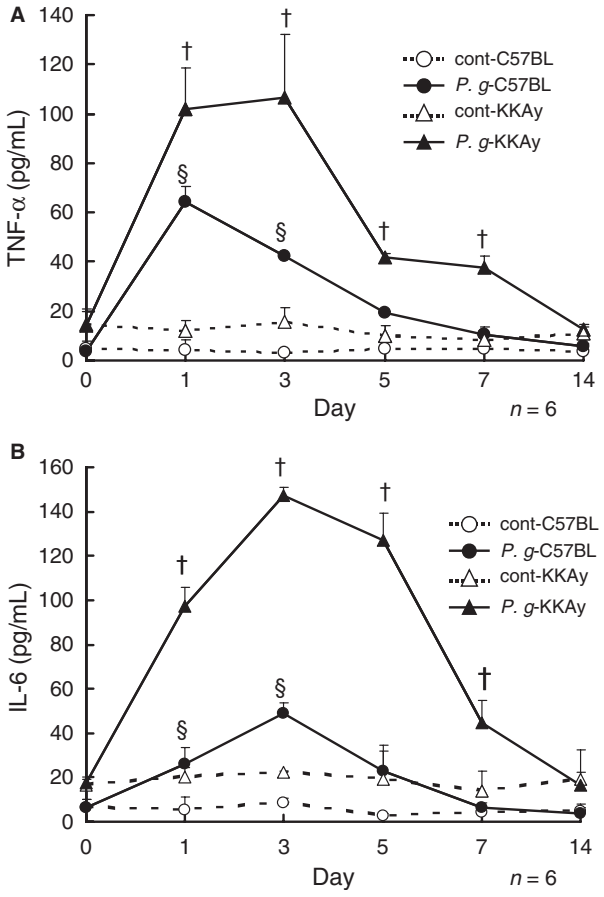

Fig. 2. Effect of inoculation with *P. gingivalis* on C57BL and KKAy mice. Peripheral blood was taken from the tail vein and the serum levels of (A) tumor necrosis factor-α (TNF-α) and (B) interleukin-6 (IL-6) were measured by enzyme-linked immunosorbent assay. †$p < 0.05$ vs. *P.g*-KKAy 0 d; §$p < 0.05$ vs. *P.g*-C57BL 0 d. cont-C57BL, control C57BL mice; cont-KKAy, control KKAy mice; *P.g*-C57BL, C57BL mice inoculated with *P. gingivalis*; *P.g*-KKAy, KKAy mice inoculated with *P. gingivalis*.

after which they gradually receded. Significantly larger abscesses were observed in the KKAy mice compared with the C57BL/6 mice on days 3, 5, 7 and 14.

The KKAy mice presented with a higher fasting blood glucose level than C57BL/6 mice. In the C57BL/6 mice, inoculation with *P. gingivalis* did not change the fasting blood glucose level. By contrast, the glucose level of the KKAy mice significantly increased after inoculation with *P. gingivalis*; the maximum level was attained on day 3, after which it steadily decreased (Fig. 1C). No significant change in body weight was observed throughout the experimental period for any of the mice (data not shown).

To explain the apparent increase in glucose concentration in the KKAy mice, we examined their serum cytokine levels using enzyme-linked immunosorbent assays. As shown in Fig. 2, both groups of mice showed a significant increase in the amounts of tumor necrosis factor-α and interleukin-6 in their serum after inoculation with *P. gingivalis*; however, significantly greater induction was observed in the KKAy mice throughout the experimental period. The maximum levels of tumor necrosis factor-α and interleukin-6 in KKAy mice were attained on day 5, after which they steadily declined.

Real-time PCR was carried out to measure the level of cytokine mRNA expression. A similar pattern of induction was observed at the mRNA level in liver of KKAy and C57BL/6 mice (Fig. 3A, B). Enhanced transcription of tumor necrosis factor-α and interleukin-6 was detected in the visceral adipose tissue of the KKAy mice (Fig. 3C, D).

The serum level of adiponectin in the KKAy and C57BL/6 mice significantly decreased to 35.7 and 58.7%, respectively, after inoculation with *P. gingivalis* (Fig. 4A). A significant reduction of adiponectin mRNA expression in visceral adipose tissue was observed throughout the experimental period, after inoculation with *P. gingivalis* (Fig. 4B).

Discussion

Periodontitis is an inflammatory condition induced by the chronic presence of periodontopathic bacteria in periodontal pockets (13,14), and cytokines are central to the initiation and maintenance of the immune response to periodontopathic bacteria. Local cytokine production in response to periodontal infection may affect the systemic environment (14,18). Moreover, these mediators can potentially increase low-grade inflammation and worsen insulin resistance (19). In our study, the expression of tumor necrosis factor-α and interleukin-6 was significantly induced by the inoculation of KKAy mice with *P. gingivalis*. Serum tumor necrosis factor-α is elevated in patients with diabetes mellitus, and tumor necrosis factor-α inhibits the activity of insulin (20,21). Tumor necrosis factor-α also inhibits insulin secretion via its effect on the β cells of the pancreas. Neutralization of tumor necrosis factor-α by soluble tumor

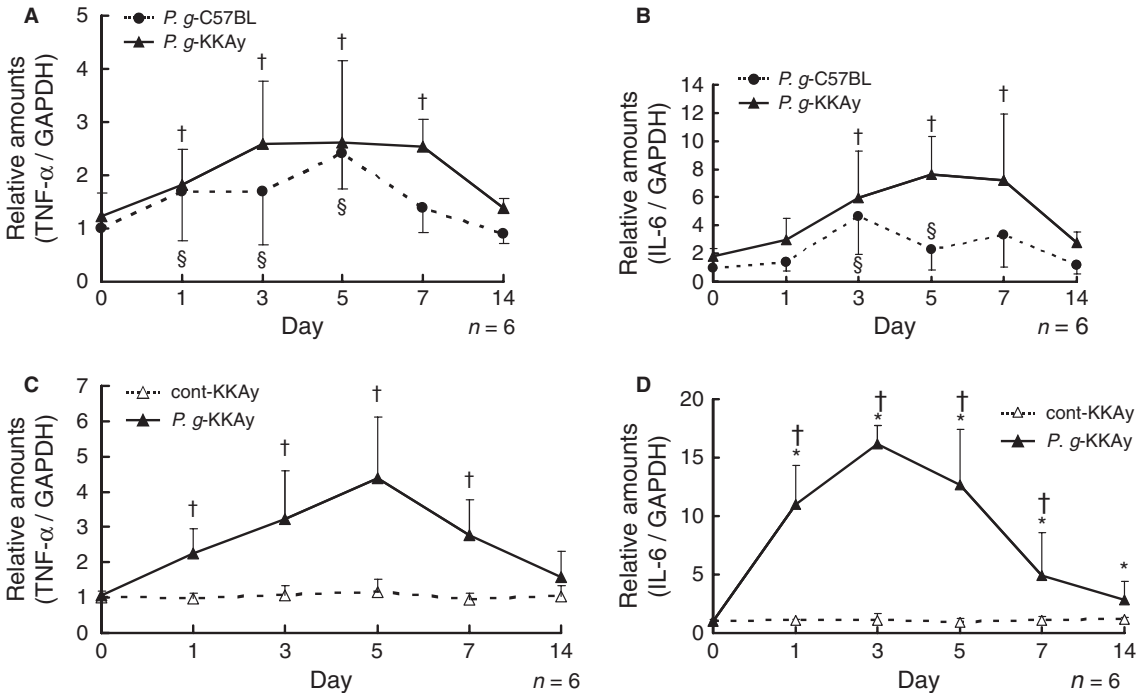

Fig. 3. Effect of *P. gingivalis* inoculation on C57BL and KKAy mice. (A) tumor necrosis factor-α (TNF-α) and (B) interleukin-6 (IL-6) mRNA expression in liver, and (C) tumor necrosis factor-α and (D) interleukin-6 mRNA expression in visceral adipose tissue were measured using real-time polymerase chain reaction. The results were normalized by reference to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). †$p < 0.05$ vs. *P.g*-KKAy 0 d; §$p < 0.05$ vs. *P.g*-C57BL 0 d; *$p < 0.05$ vs. *P.g*-KKAy. cont-C57BL, control C57BL mice; cont-KKAy, control KKAy mice; *P.g*-C57BL, C57BL mice inoculated with *P. gingivalis*; *P.g*-KKAy, KKAy mice inoculated with *P. gingivalis*.

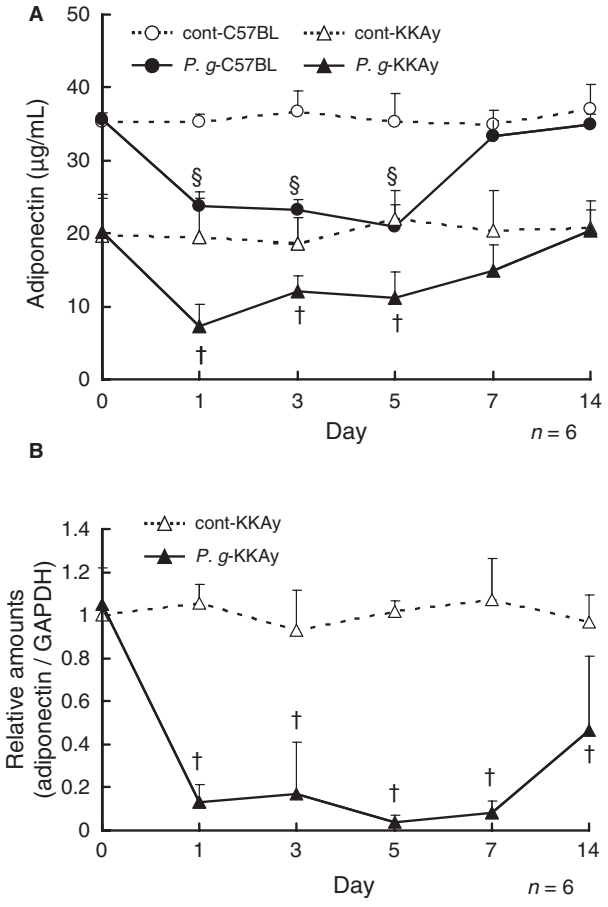

Fig. 4. Changes in the adiponectin level after inoculation with *P. gingivalis*. (A) The level of adiponectin in serum was measured using an enzyme-linked immunosorbent assay. (B) RNA was extracted from visceral adipose tissue, and the expression of adiponectin was measured by real-time polymerase chain reaction. The results were normalized by reference to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). †$p < 0.05$ vs. *P.g*-KKAy 0 d; §$p < 0.05$ vs. *P.g*-C57BL 0 d. cont-C57BL, control C57BL mice; cont-KKAy, control KKAy mice; *P.g*-C57BL, C57BL mice inoculated with *P. gingivalis*; *P.g*-KKAy, KKAy mice inoculated with *P. gingivalis*.

necrosis factor-α receptor was found to reduce insulin resistance in an animal model (22). An increased level of interleukin-6 in the circulation is also associated with insulin resistance in humans (23). Longitudinal prospective studies suggest that the serum level of interleukin-6 can be used to predict the occurrence of diabetes mellitus (24). Our study indicated that one major source of the increased levels of tumor necrosis factor-α and interleukin-6 in KKAy mice may be adipose tissue, and the prolonged local inflammatory response of KKAy mice to *P. gingivalis* might play a crucial role in the induction of tumor necrosis factor-α and interleukin-6 in adipose tissue. These observations indicate that tumor necrosis factor-α and interleukin-6 may be important factors in the bidirectional inter-relationship between diabetes mellitus and periodontitis.

Adipose tissue has been increasingly recognized as an important endocrine organ that secretes several mediators (22,25). Of these, adiponectin has attracted much recent attention because of its antidiabetic and antiatherogenic effects, and it is expected to be useful as a therapeutic agent for diabetes (26). Several studies have examined the plasma adiponectin level in humans and found decreased levels in diabetic subjects with significant inverse associations with some measure of insulin resistance (27,28). Adiponectin suppresses tumor necrosis factor-α production in adipose tissue and hence improves insulin sensitivity (29). Adiponectin also reduces the production and activity of tumor necrosis factor-α in monocytes and macrophages. The anti-inflammatory activities of adiponectin extended to the inhibition of interleukin-6 production accompanied by the induction of anti-inflammatory cytokines, such as interleukin-10 (30,31). In our study, the inoculation of *P. gingivalis* into KKAy and C57BL/6 mice significantly reduced the expression of adiponectin, which may lead to a synergistic action on inflammatory activities. Tumor necrosis factor-α and interleukin-6 were shown to down-regulate adiponectin synthesis in adipocytes (32). Therefore, the induction of tumor necrosis factor-α and interleukin-6 by inoculation with *P. gingivalis* may at least partly suppress adiponectin expression in serum and visceral adipose tissue. Moreover, reduced adiponectin levels also directly play a casual role in the development of atherosclerosis (33). Indeed, a decrease in the serum level of adiponectin by genetic and environmental factors has been shown to contribute to the development of cardiovascular disease. Adiponectin reduces induction of the endothelial adhesion molecules intercellular adhesion molecule-1 and vascular cell adhesion molecule (33,34). Inhibition of adhesion molecules by adiponectin might explain the development of atherosclerosis. These observations indicate that reduced adiponectin levels in *P. gingivalis* infection may play an important role in the development of insulin resistance, diabetes and atherosclerosis in patients with periodontitis. Moreover, a recent study showed that adiponectin increases bone mass by suppressing osteoclast function and activating osteoblasts (35). Reduced adiponectin levels may also play a role in the initiation and progression of alveolar bone destruction in patients with periodontitis.

In conclusion, infection with *P. gingivalis* significantly induced the expression of tumor necrosis factor-α

and interleukin-6 in KKAy mice. By contrast, the level of adiponectin decreased following inoculation with *P. gingivalis*. Our data suggest that *P. gingivalis* infection may be an important risk factor for diabetes, although the role of periodontal inflammation in diabetes mellitus is inconclusive.

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