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Susceptibility of type 2 diabetic mice to low-virulence bacterial infection: induction of abscess formation by gingipain-deficient *Porphyromonas gingivalis*

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Background and Objective: Type 2 diabetes mellitus is considered an important risk factor of adult periodontitis. However, recent studies have revealed that the subgingival microbial flora of diabetes mellitus patients does not differ from that of healthy individuals. In this study, we examined the response of type 2 diabetes mellitus hosts to low-virulence bacteria in a murine abscess model.

Material and Methods: *Porphyromonas gingivalis* ATCC 33277 or KDP128 (*rgpA rgpB kgp*) were injected into two mouse strains – C57BL/6J and its derivative, KK/A^{Y}, which becomes diabetic spontaneously.

Results: Lesions of KK/A^{Y} mice injected with either low-virulence *P. gingivalis* KDP128 or wild-type 33277 were significantly larger than those of C57BL/6J mice injected with the same strains. Histologically, more neutrophils and macrophages migrated to the lesions in the KK/A^{Y} mice injected with *P. gingivalis* 33277 and KDP128 compared with those of C57BL/6J mice injected with the same respective strains.

Conclusion: These results suggest that severe inflammation is observed in response to low-virulence bacteria in addition to the highly virulent bacteria in type 2 diabetes mellitus hosts.

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Diabetes mellitus is considered to be an important risk factor of periodontitis (1–4), and significant attachment loss and periodontal bone resorption has been reported in patients with diabetes mellitus (5–7). Type 1 diabetes mellitus is caused by destruction of β cells in the pancreas, whereas type 2 diabetes mellitus results from defects in the insulin molecule or from altered cell receptors for insulin, manifesting in impaired insulin function. About 90% of diabetes mellitus patients belong to the latter type. Suppressed chemotactic and phagocytic activities of neutrophils are thought to be related to severe periodontal breakdown in diabetes mellitus patients (8). Macrophages of

diabetes mellitus patients also secrete increased levels of inflammatory cytokines, such as tumor necrosis factor-α, prostaglandin E2 and interleukin-1β, which play an important role in both host defense and periodontal breakdown (9).

Recent studies have indicated that the subgingival microflora of diabetes mellitus patients is not different from that of healthy individuals (10). These results suggest that diabetic hosts are more susceptible to periodontal bacteria, including low-virulence bacteria. To confirm this hypothesis, we compared the host response of normal and diabetes mellitus mice to bacteria with different relative pathogenic properties.

Porphyromonas gingivalis is thought to be one of the most important periodontopathogens (11,12). *P. gingivalis* has many biologic activities, and gingipains are known to be associated with numerous pathological functions (13,14). We previously reported that mutant strains of *P. gingivalis*, devoid of gingipain activities, showed less virulence in a murine abscess model (15). In the present study we examined the effects of low-virulence bacteria on the type 2 diabetic host. We also compared the responses of inflammatory cells against different bacterial strains in normal and diabetic mice.

Material and methods

Animals

The two mouse strains, C57BL/6J and KK/A^Y (5 wk old, female), were obtained from Nippon Clea Co. (Tokyo, Japan). KK/A^Y mice are generated from C57BL/6J mice and are known to be obese and to initiate a diabetic condition spontaneously. Ten mice were used in each experimental group. They were housed in isolator cages in the animal facility of the Kyushu University Faculty of Dental Science and were maintained on a 12-h light/dark cycle and received water and food *ad libitum*. We observed the general body condition and measured the body weight of all mice daily. Once a week, peripheral blood was taken from the tail vein and the fasting plasma glucose levels were measured using a glucose measuring device (GT-1640; SKK Co. Ltd, Osaka, Japan). At the same time, we measured the glucose levels in urine using Tes-tape (Shionogi Co. Ltd, Osaka, Japan). If glucose was detected in the urine, the mouse was considered diabetic. The experiment was started after confirming that all KK/A^Y mice were diabetic and all C57BL/6J mice were not.

Bacterial strains and growth conditions

P. gingivalis ATCC 33277 and KDP128 (*rgpA rgpB kgp*) (16), which were kindly provided by K. Nakayama., were used in these experiments. Bacteria were maintained on CDC anaerobic blood agar (Becton Dickinson, Cockeysville, MD, USA), in an anaerobic atmosphere (80% N_2, 10% H_2, 10% CO_2), and inoculated in brain heart infusion broth supplemented with hemin (5 μg/mL) and menadione (1 μg/mL). Erythromycin (10 μg/mL) was used for culturing KDP128 when necessary.

Murine abscess model

The abscess-formation experiments were conducted as reported previously (15). *P. gingivalis* ATCC 33277 or KDP128 were grown in liquid medium to the late logarithmic phase, harvested by centrifugation, and gently resuspended in brain heart infusion broth. Absorbance of the cell suspensions was measured at 600 nm, and cell concentrations were adjusted to 3.6×10^{10}/mL. Mice were subcutaneously injected at two sites in the lateral dorsal region with 200 μL (100 μL per site) of bacterial suspension. The two injection sites were at least 2.0 cm apart to prevent fusion. Forty mice were distributed into the following four groups ($n = 10$ per group): KK/A^Y mice injected with strain ATCC 33277 (KK/A^Y–33277), KK/A^Y mice injected with strain KDP128 (KK/A^Y–128), C57BL/6J mice injected with strain ATCC 33277 (C57–33277), and C57BL/6J mice injected with strain KDP128 (C57–128). The sizes of the subcutaneous abscesses or necrotic skin lesions were measured using a caliper gauge, and lesion sizes were expressed in mm^2. These examinations were performed once a day during the 14-d postinfection period.

Histological evaluation

Three mice in each group were killed on day 5 when abscesses were apparently induced. Tissues including the whole abscess were dissected and fixed by perfusion with 4% paraformaldehyde in 0.05 M phosphate-buffered saline, pH 7.5, at 4°C for 24 h. After dehydration with ethanol, specimens were embedded in paraffin and then sliced serially at 4-μm intervals. After deparaffinization, the sections were stained with hematoxylin and eosin, and light microscopic observations were performed. The sections were also examined immunohistochemically with rat antimouse F4/80 antigen (MCA497; Serotec Ltd, Oxford, UK) diluted 1 : 300 in phosphate-buffered saline. After using the Histofine Simple Stain Mouse MAX-PO kit (Nichirei Co. Ltd, Tokyo, Japan), the sections were incubated in diaminobenzidine-H_2O_2 solution (Nichirei Co. Ltd), and then counterstained with 3% methyl green (Waco Co. Ltd, Osaka, Japan). The number of macrophages infiltrating around the abscesses was counted using the reticule eyepiece enclosing a 7×7 grid (Olympus, Tokyo, Japan) at ×200 magnification. The area covered by the reticule eyepiece was 0.000615 mm^2 under these conditions. Counting was performed three times for each site around the lesions.

Statistical analysis

Data in the figures are expressed as the mean and standard error of more than eight lesions. Significance was evaluated using a two-tailed unpaired *t*-test with STATVIEW 5.0 software for Macintosh.

Results

General animal conditions

Animal body conditions at the start of the experiment are shown in Table 1. Serum and urinary glucose levels and body weights of KK/A^Y mice were

Table 1. Animal conditions at the start of the experiment

Injecting bacteria	Mouse strain	Blood glucose level[a]	Urinary glucose level[b]	Body weight[c]
P. gingivalis ATCC 33277	KK/Ay	306 (24.3)	+ + +	45 (1.4)
	C57BL/6J	147 (5.37)	–	20 (0.18)
P. gingivalis KDP128	KK/Ay	416 (25.9)	+ + +	50 (0.34)
	C57BL/6J	183 (11.0)	–	21 (0.34)

[a]GT-1640 score (mg/dL ± SE).
[b]Tes-tape score (–, +, + +, + + +).
[c]g ± SE.

higher than those of C57BL/6J mice, but there was no significant difference between each strain injected with ATCC 33277 or KDP128. No mice showed significant body weight gain or loss during the experimental period.

Time course of lesion formation

The time course of lesion formation in each group is shown in Fig. 1. When *P. gingivalis* ATCC 33277 was injected into C57BL/6J mice (C57-33277), soft round abscess formation was observed. Three days after bacterial challenge, clear abscesses were induced, and they maintained their size until day 8. Then they ulcerated and their sizes were gradually reduced. When the same bacterial suspension was inoculated into KK/A^Y mice (KK/A^Y–33277), much larger abscesses were induced. The abscesses reached their maximum size early (day 4), after which they ulcerated and rapidly decreased in size but were still larger than those of the other groups throughout the experimental period. When the mutant strain of *P. gingivalis* (KDP128, devoid of gingipain activities) was injected into C57BL/6J mice (C57–128), no clear abscesses were induced, and only swelling was observed. When this mutant strain was inoculated into KK/A^Y mice (KK/A^Y–128), clear abscesses were induced gradually and the maximum size was maintained throughout the experimental period.

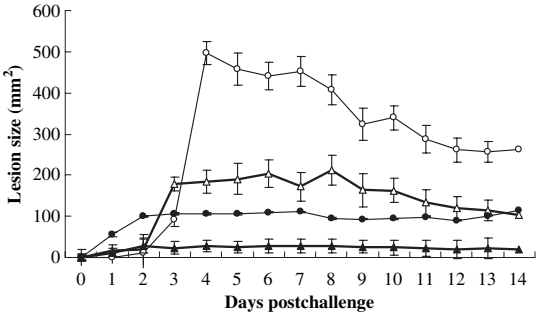

Fig. 1. Dynamics of abscess formation induced by *Porphyromonas gingivalis* ATCC 33277 or KDP128. *P. gingivalis* ATCC 33277 or KDP128 (7.2×10^9 cells per mice) was injected into each mouse strain. ○, KK/A^Y injected with ATCC 33277; △, C57BL/6J injected with ATCC 33277; ●, KK/A^Y injected with KDP128; and ▲, C57BL/6J injected with KDP128. After injection of each bacterium, lesion sizes were measured once a day.

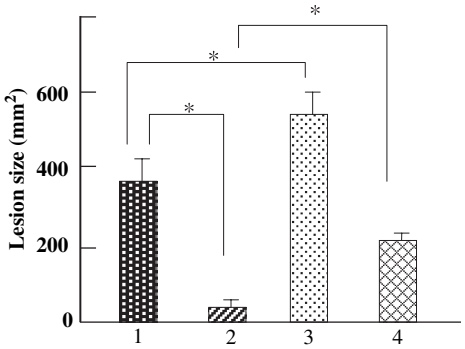

Fig. 2. Maximum lesion sizes induced by *Porphyromonas gingivalis*. The maximum lesion sizes of each mouse group during the experimental period were compared. Column 1, C57BL/6J–*P. gingivalis* ATCC 33277; column 2, C57BL/6J–KDP128; column 3, KK/A^Y–*P. gingivalis* ATCC 33277; and column 4, KK/A^Y–KDP128. *Significant difference in triplicate assays at $p < 0.01$.

Comparison of the maximum lesion area in each group

The maximum lesion sizes in each group are summarized in Fig. 2. The lesion size of the KK/A^Y–33277 group was significantly larger than that of the C57–33277 group. Very small lesion formation was observed in the C57–128 group. On the other hand, the lesion size of the KK/A^Y–128 group was significantly larger than that of the C57–128 group ($p < 0.01$).

Histological evaluation

In the C57–33277 group, large lesions with infiltrating neutrophils were observed (Fig. 3A). Abscesses were widely surrounded by strikingly loose tissues. In the C57–128 group, only microabscesses were observed, which were surrounded by dense connective tissue. The infiltration of neutrophils was localized around the lesions (Fig. 3B).

In the KK/A^Y–33277 group and the KK/A^Y–128 group, remarkable neutrophil infiltration was seen in and around the lesions (Fig. 3C,D). Abscesses in the KK/A^Y–33277 group were surrounded by loose tissue and irregularly arranged fibers (Fig. 3C), but in the KK/A^Y–33277 group, abscesses were continuously encapsulated by fibrous tissues (Fig. 3D).

Immunohistological evaluation

We also examined the abscesses by immunohistological staining with

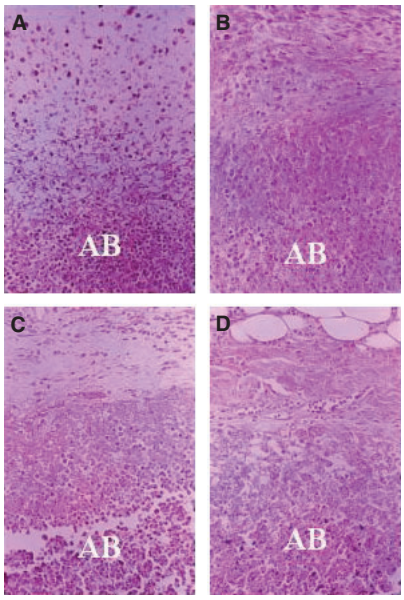

Fig. 3. The results of hematoxylin and eosin staining (magnification ×400). AB, internal area of the lesion. (A) Lesion of a C57BL/6J mouse injected with *Porphyromonas gingivalis* ATCC 33277. An abscess was widely surrounded by strikingly loose tissues. (B) Lesion of a C57BL/6J mouse inoculated with KDP128. The infiltration of neutrophils was localized and surrounded by dense connective tissues. (C) Lesion of a KK/A^{Y} mouse inoculated with *P. gingivalis* ATCC 33277. An abscess was surrounded by loose tissues and irregularly arranged fibers. (D) Lesion of a KK/A^{Y} mouse inoculated with KDP128. An abscess was continuously encapsulated by fibrous tissues.

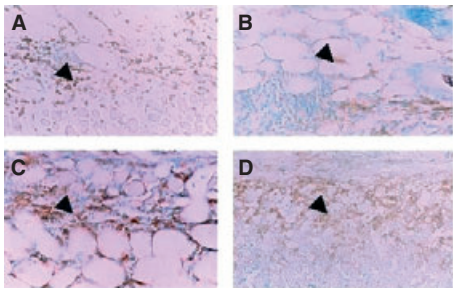

Fig. 4. Immunostaining with F4/80 (magnification, ×400). Arrowheads indicate macrophages. (A) Lesion of a C57BL/6J mouse inoculated with *Porphyromonas gingivalis* ATCC 33277. Large numbers of macrophages were observed around the lesion, but the surrounding tissue was coarse. (B) Lesion of a C57BL/6J mouse inoculated with KDP128. Small numbers of macrophages were observed adjacent to the lesion. (C) Lesion of a KK/A^{Y} mouse inoculated with *P. gingivalis* ATCC 33277. Large numbers of macrophages were observed around the lesion. (D) A lesion of a KK/A^{Y} mouse inoculated with KDP128. Large numbers of macrophages were observed around the lesion.

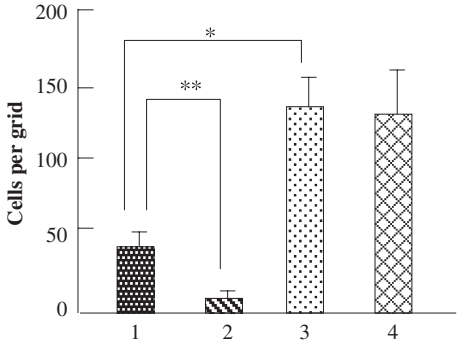

Fig. 5. The number of F4/80-positive cells. The number of macrophages was counted per grid around the lesion. Column 1, C57BL/6J–*Porphyromonas gingivalis* ATCC 33277; column 2, C57BL/6J–KDP128; column 3, KK/A^{Y}–*P. gingivalis* ATCC 33277; and column 4, KK/A^{Y}–KDP128. Cell counting was performed three times with each sample. Significant difference between two columns were observed at $*p < 0.05$ and $**p < 0.01$.

antimouse macrophage F4/80 antigen. Macrophages were observed around the lesions of all specimens, but their distribution and localization differed among experimental groups. In the C57–33277 group, round or oval-shaped macrophages were seen infiltrating around the abscesses (Fig. 4A). In the C57–128 group, only small numbers of macrophages were observed around localized lesions (Fig. 4B). In the KK/A^{Y}–33277 group, large numbers of macrophages were observed (Fig. 4C). Morphologically they showed various shapes, such as oval, elongate and polygonal. Moreover, the infiltration of macrophages was enlarged to the adipose tissue at a distance from the lesion (Fig. 4C). In the KK/A^{Y}–128 group, large numbers of macrophages were also observed around the lesions (Fig. 4D).

Histometric evaluation

The numbers of macrophages infiltrating the abscess area were counted (Fig. 5). There were significantly more macrophages in the C57–33277 group than in the C57–128 group ($p < 0.01$). There were also significantly more macrophages in the KK/A^{Y}–33277 group than in the C57–33277 group ($p < 0.01$). However, there was no significant difference between the KK/A^{Y}–33277 and KK/A^{Y}–128 groups.

Discussion

Evanthia *et al.* previously reported that alveolar bone loss induced by *P. gingivalis* is significantly accelerated in type 1 diabetes mellitus mice (17). They also reported that alveolar bone resorption was induced in the type 1 diabetes mellitus mice treated with phosphate-buffered saline only. Taking into consideration that the experiment was not performed under bacteria-free conditions, indigenous or low-virulence bacteria may have induced inflammation in the periodontium of diabetes mellitus mice.

Murine abscess models are frequently used to determine the virulence of bacteria (18). We had previously reported that the abscess-forming activity of the *P. gingivalis* strains, devoid of gingipain activities, is significantly decreased (15,19). We used this strain to establish the effect of low-virulence bacteria on the host

response of the type 2 diabetes mellitus mouse.

When the wild-type strain *P. gingivalis* 33277 was injected into C57BL/6J mice (normal/wild-type combination), large abscesses were induced, but no clear abscesses were observed when the gingipain mutant was injected into the same mouse strain (normal/mutant combination). These findings are consistent with our previous report (15), which showed the importance of gingipains in the abscess formation in Balb/cN mice.

On the other hand, when the *P. gingivalis* strain, devoid of gingipain activities, was injected into the diabetes mellitus mouse, clear abscesses were induced. These results suggest that low-virulence bacteria can also exhibit pathogenicity in diabetes mellitus hosts. This is also consistent with the epidemiological findings that diabetes mellitus patients experience serious periodontitis (3,20).

From the results of this report, we cannot exclude the possibility that the accelerated inflammation was caused by obesity, rather than diabetes mellitus itself. In our preliminary experiment, we used obese nondiabetic KK/Ta mice (bodyweight, 40 ± 0.4 g; blood glucose, 127 ± 19 mg/dL; urinary glucose, –). The gingipain-deficient strain of *P. gingivalis* induced lesion formation on the KK/Ta mice, but the lesions were not as large as those of KK/A^Y mice. This suggested that glycemic control might be more important in the host response to the bacterial invasion. To clarify this point, we are planning to use nonobese diabetes mellitus mice in future experiments.

There are many reasons for the severe breakdown of periodontal tissues in diabetes mellitus hosts. Migration of inflammatory cells into peripheral tissues might contribute to this breakdown.

When wild-type or a gingipain mutant strain of *P. gingivalis* was injected into the diabetes mellitus mice, inflammatory cells, mainly consisting of neutrophils, were seen in abundance in and around the abscesses. This is consistent with the clinical findings that more neutrophils migrate to the crevicular sulcus of diabetes mellitus patients, especially those whose blood glucose levels are poorly controlled (21). The migrated neutrophils may also contribute to the enhancement of inflammation via the generation of superoxides (22).

More macrophages also migrated to the abscesses when wild-type *P. gingivalis*, or a gingipain mutant strain of *P. gingivalis*, was injected into the diabetes mellitus mice. The numbers of migrated macrophages did not differ between diabetes mellitus/wild-type and diabetes mellitus/mutant combinations, indicating that even a low-invasive bacterial strain can induce inflammatory responses in the diabetes mellitus host. This is also consistent with the clinical findings that the chemotactic activity of monocytes in the peripheral blood of diabetes mellitus patients is activated and the monocytes release excessive amounts of pro-inflammatory cytokines (23–25). Monocytes of the type 1 and type 2 diabetes mellitus hosts are known to produce more tumor necrosis factor-α (26,27), prostaglandin E2 and interleukin-1β (28) by stimulation with *P. gingivalis* lipopolysaccharide compared with normal hosts. Therefore, in the type 2 diabetes mellitus mice, macrophages are considered to play an important role in the inflammatory response to less virulent bacteria.

Another reason for severe abscess formation in diabetes mellitus mice could be the disturbance of host defense mechanisms, as reported in humans (8). Thus, more bacteria may be growing in the diabetes mellitus mice. It will be important to monitor bacterial growth and humoral immune responses after injection of bacteria, and this is now in progress in our laboratory. Further work is obviously necessary to determine the relationship between diabetes mellitus and periodontitis, and the murine abscess model may contribute to progress in this field of research.

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