The effect of leukocyte function of streptozotocin-induced diabetes in naturally occurring gingivitis rat

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Although diabetes mellitus is known to aggravate periodontal disease, the precise relationship between these two entities is far from being completely understood. Further study of this relationship was therefore undertaken in the form of observation of both naturally occurring gingivitis in rats (ODUS/Odu) and effects produced by induction of experimental diabetes mellitus by injection of streptozotocin (STZ: 65 mg/kg, i.v.). At one and 3 mon after STZ injection, liquid paraffin was injected intraperitoneally. Four days thereafter, pocket probing depths of rats were measured and blood samples as well as peritoneal macrophages were collected from both experimental animals and non diabetic controls. Both chemotaxis and phagocytosis of macrophages were studied. At one and 3 mon after STZ injection, pocket probing depths of diabetic animals were significantly deeper than those of controls (p<0.001). Pocket probing depths were deeper at 3 mon after STZ injection than after 1 mon in diabetic animals. At three months after STZ injection, there was a high degree of positive correlation between pocket probing depths, blood glucose levels, triglyceride, and hemoglobin A1c levels (p<0.01). Also, macrophage chemotaxis was more suppressed in diabetic rats than it was in controls. Additionally, both phagocytosis ratios and phagocytosis indices of macrophages in the diabetes group were significantly more suppressed than those in controls in both experimental periods (p<0.001). Findings suggest that both chemotaxis and phagocytosis are compromised in macrophages from rats rendered diabetic by STZ injection. Thus as host defense mechanisms become weakened, there is a corresponding progression of periodontal disease. (J Osaka Dent Univ 1997 Dec; 31: 47-54.)

Key words: Diabetes mellitus, Macrophage, Chemotaxis, Phagocytosis, Periodontal disease

INTRODUCTION

It has been known that diabetic patients are more likely to develop diseases with exaggerated and severe symptoms owing to overall diminished defense mechanisms. Rapid progression of periodontal disease in the diabetic is one such dental example.1-12 In the diabetic patients are often resistant to standard forms of treatment, researchers for sometime have sought to delineate the cause or causes of intractable immunocompromise in diabetics13,14 in hopes that results would lead to more effective periodontal therapy. To date, however, helpful conclusive results remain elusive.

We considered that inhibition of leukocyte protection against nonspecific infection in diabetics might represent one of the factors that contribute to intractability of periodontal diseases. Accordingly, we undertook a study to investigate this possibility employing rats in which diabetes mellitus was experimentally induced using streptozotocin (STZ) injections.15-19 Leukocytes were subsequently harvested from the peritoneal cavity and functionally analyzed in order to estimate the possible contributory effects
of compromised inflammatory cells on periodontal disease.

**MATERIALS AND METHODS**

**Experimental animals**
Thirty-three male naturally occurring gingivitis in rats (ODUS/Odu) weighing 180–220 g (2 mon old) were used in this study. One group (N=23) was injected with streptozotocin (STZ : 65 mg/kg, i.v., Sigma Chem. Co., St Louis, MO, USA) dissolved in 0.05 M citrate buffer (pH 4.5), and animals were assessed as diabetic if subsequent blood glucose level rose to level higher than 300 mg/dl. The control group (N=10) was injected with citrate buffer alone.

**Measurement of pocket probing depths**
Pocket probing depths were probed and measured at the center of each lower incisor using a WHO recommended probe. The mean of two measurements of pocket probing depth was calculated.

**Determination of blood glucose levels**
Blood glucose levels were quantified using the mutarotase-glucose oxidase method (Glucose C-test Wako : Wako Pure Chemical Industries, Ltd., Osaka, Japan).

**Determination of serum insulin levels**
Serum insulin levels were determined by the one step enzyme immunologic assay (Glazyme insulin-EIA TEST : Wako Pure Chemical Industries, Ltd.)

**Determination of hemoglobin A1c levels in serum**
The amount of hemoglobin A1c in serum was quantified using Roopet A1c-S (Nippon Chemiphar Co., Ltd., Tokyo, Japan).

**Determination of triglycerides in serum**
The amount of triglyceride in serum was quantified using Triglyceride E-test Wako (Wako Pure Chemical Industries, Ltd.).

**Preparation of rat macrophages**
Sterilized liquid paraffin (10 ml/100 g body weight, Matsumura Oil Research Co., Japan) was injected peritoneally, and after 4 days, peritoneal macrophages were harvested from both control and experimental groups. Cells were then washed twice in 20 mM phosphate buffered saline (PBS : pH 7.2) and resuspended in Dulbecco’s Modified Eagle Medium (DMEM : Gibco-BRL, USA) with streptomycin sulfate (2 mM : Gibco-BRL) and penicillin G sodium (100 U/ml : Gibco-BRL).

**Chemotactic stimulus and phagocytes**
Zymosan activated serum (ZAS) was used as stimulant of macrophage chemotaxis and opsonized zymosan (OPZ) served as substrate for macrophage phagocytosis. ZAS was obtained by the following method. Zymosan A (40 mg/ml : Sigma Chemical Co.,) derived from Saccharomyces cerevisiae, was suspended in saline, and incubated at 100°C for 10 min. It was then washed and resuspended in saline. Fresh serum was then isolated from Wistar rats and incubated at 37°C for 30 min with the same volume of zymosan A as above. After centrifugation (3,000 rpm, 10 min), the supernatant was incubated at 56°C for 30 min, then filtered using a 0.45 μm filter, and stored at −20°C until use. OPZ was prepared as above using the sediment after washing with cold saline. The latter was then resuspended in 20 mg/ml of Earle’s Balanced Salt Solution (EBSS) and stored until use.

**Chemotaxis assay**
Macrophage migration was measured by the membrane filter method using a 48-well micro chemotaxis chamber (Neuro Probe Inc., USA). Samples were diluted with a mixture of seven parts Gey’s balanced salt solution (GBSS : Gibco Life Technologies Inc., New York, USA), 2% bovine serum albumin (Gibco Life Technologies Inc.,) 20 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (Sigma Chemical Co.,) Gey’s BSA, pH 7.2) and five parts of Veronal buffer with 2 M MgCl2 and 1 M CaCl2(VB2). This mixture is referred to as Gey’s BSA-VB2. Using a micro chemotaxis chamber, the macrophage density was determined to be 2×10⁶
cells/ml in Gey’s BSA. The cell suspension was then placed in the upper wells of the chamber separated by a polycarbonate membrane filter with 5 μm pores (Nuclepore: Costar Corp., USA). The lower wells contained ZAS as the chemotactic stimulant or Gey’s BSA-VB² as a negative control. Following incubation for 90 min in a humidified atmosphere containing 5% CO₂, the filters were removed, fixed, stained and mounted on slides and viewed through a microscope. Macrophages migrating to the lower surface of the filter were counted automatically (Mac SCOPE: MITANI Corp., Osaka, Japan). Data represent the mean calculation of macrophages per oil immersion field using triplicate filters.

**Phagocytosis assay**

Cell suspension were placed in multi-well glass slides and preincubated for 60 min under the same conditions as the chemotaxis assay. Glass slides were then washed twice with PBS. Most cells adherent to the glass slides after preincubation were macrophages with a density of 2 × 10⁵ cells/well. OPZ was added in DMEM for phagocytosis, and the glass slides were incubated under the same conditions as the preincubation process. Macrophages were then washed twice with PBS to abolish phagocytosis, dried and dyed with Giemsa’s stain (Merck Japan, Japan). Both the number of cells and phagocytosed particles were counted using an optical microscope. Phagocytosis ratios (PR) were determined as the percent of ingesting cells whereas the phagocytosis index (PI) was determined as the average number of ingested particles per cell counting the same 100 cells as used to determine the PR. Both PR and PI were expressed algebraically as follows:

1. \( PR = \frac{\text{Number of ingesting cells}}{\text{Number of counted cells}} \times 100\% \)
2. \( PI = \frac{\text{Number of ingested OPZs}}{\text{Number of counted cells}} \)

**Statistical analysis**

Data shown are the calculated means and standard error. The statistical significance of the data was determined by the Student’s t-test. A probability value (P) of less than 0.05 was considered to be significant.

**Approval by the animal welfare committee**

The use of experimental animals in this study was approved by the Animal Welfare Committee of Osaka Dental University.

**RESULTS**

**Blood glucose levels**

Mean blood glucose levels were as follows. At 1 month following injections, blood glucose levels of ODUS/Odu with and without diabetes measured 460 ± 18 mg/dl and 95 ± 12 mg/dl, respectively while at 3 months, respective levels were 468 ± 16 mg/dl and 93 ± 10 mg/dl (Fig. 1). Clearly, mean blood glucose levels at both 1 and 3 months in ODUS/Odu induced diabetes were significantly higher than those of the control group (p<0.001, Fig. 1).

**Blood insulin levels**

Blood insulin levels at both 1 and 3 months in ODUS/Odu with induced diabetes were significantly lower than that of the control group (p<0.001, Fig. 2).

**Pocket probing depths**

![Graph showing changes in pocket probing depths](image)

**Fig. 1** Changes in blood glucose levels in ODUS/Odu with and without diabetes. Data are shown as mean ± S.E. At 1 and 3 months after STZ injection, levels are significantly higher than those of the control group (p<0.001).
Pocket probing depths at both 1 and 3 mon in ODUS/Odu with induced diabetes were significantly deeper than those of the control group (p < 0.001). Pocket probing depths in ODUS/Odu with diabetes 3 mon following STZ injection were deeper than those of 1 mon (Fig. 3).

**Correlation between pocket probing depths and blood glucose levels**

At both 1 and 3 mon after STZ injection, there was a direct positive correlation between blood glucose levels and pocket probing depths of animals with and without experimentally induced diabetes (Y = 0.005X + 1.43, r = 0.85, p < 0.01, Fig. 4).

**Correlation between pocket probing depths, triglycerides and hemoglobin A1c levels**

At 1 and 3 mon after STZ injection, there was a di-
Fig. 6 Relationship between pocket probing depths and hemoglobin A_1C levels at 1 and 3 mon after STZ injection in ODUS/Odu. At the both periods after STZ injection, a positive correlation was found between hemoglobin A_1C levels and pocket probing depths.

Table 1 Effects of streptozotocin (STZ) induced diabetes mellitus on macrophage chemotaxis in ODUS/Odu in zymosan activated serum (ZAS)

<table>
<thead>
<tr>
<th>ZAS</th>
<th>ODUS/Odu with diabetes</th>
<th>ODUS/Odu without diabetes</th>
<th>Inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:20</td>
<td>594 ± 30°</td>
<td>722 ± 18</td>
<td>18</td>
</tr>
<tr>
<td>1:40</td>
<td>524 ± 36°</td>
<td>696 ± 17</td>
<td>25</td>
</tr>
<tr>
<td>1:80</td>
<td>529 ± 17°</td>
<td>658 ± 27</td>
<td>20</td>
</tr>
</tbody>
</table>

Three months after STZ injection. The results are shown as mean ± S.E. and statistical analysis was carried out by Student's t-test. Significance was *p < 0.01 and *p < 0.05 compared to the positive control of macrophage chemotaxis of ODUS/Odu without diabetes (the control group).

Fig. 7 Phagocytosis ratio and experimental period of ODUS/Odu with and without diabetes. Data are shown as mean ± SE at 1 and 3 mon after STZ injection, ratios are significantly suppressed compared to the control group (p < 0.001).

Macrophage migration in rats
As shown in Table 1, there was suppressed ZAS induced chemotaxis in macrophages of experimental diabetic animals 3 mon following STZ injections. ODUS/Odu with diabetes showed suppressed chemotaxis compared to the macrophages from the control group. However, there was no significant inhibition of macrophages chemotaxis of 1 mon

ODUS/Odu with diabetes compared to the control group. Random migration did not differ between the two groups (data not shown).

Macrophage phagocytosis ratios and phagocytosis indices in rats
At both 1 and 3 mon after STZ injection, macrophage phagocytosis ratios from ODUS/Odu with
diabetes differed significantly from those of the control group (p<0.001, Fig. 7). In addition, the phagocytosis indices of diabetic animals versus controls was similarly suppressed (p<0.001, Fig. 8).

**DISCUSSION**

Diabetes mellitus is a complex metabolic disease manifest primarily by a relative or absolute deficiency of insulin. Generally, diabetes is classified into either primary or secondary diabetes mellitus with primary diabetes mellitus being further classified into either insulin-dependent, type I or non-insulin dependent, type II forms.

STZ as used in this study destroys β-cells in the islets of Langerhans thus inducing an artificial insulin deficiency that fulfills criteria of experimental type I diabetes mellitus. When type I or type II diabetic hyperglycemia remains untreated humans, vascular disorders develop leading to diabetic retinopathy or nephropathy. Such patients also develop a variety of chronic complications peculiar to diabetes mellitus. Some of these are manifest by clinical symptoms or pathological changes within the oral cavity\(^1\,\!\!\!^2\) including changes in blood vessels, shifts in bacterial populations, altered biological defense mechanisms (leukocyte insufficiency), disturbed collagen metabolism and impaired wound healing.

Continuous diabetic hyperglycemia is thought to induce angiopathy by promoting glycosylation of basement membrane proteins independent of enzymes (HbA\(_{1c}\) is produced by the binding of glucose and hemoglobin as a result of mutual reaction between sugar and amino acids, and it is known that the level of HbA\(_{1c}\) increases in diabetic patients\(^1\)\!\!\!^2\). Similar vascular changes can be observed in all tissues, and it is suggested that such vascular changes are responsible not only for inducing eating disorders but also for inhibiting transportation of metabolites and chemotaxis of leukocytes to gingival tissues thus exacerbating more aggressive periodontal diseases. Also, since collagen is the main gingival and alveolar bone structural component (accounting for roughly 60% of gingival connective tissues), increased collagenase activity observed in diabetics is thought to play a role in both the rapid progression of periodontal diseases and delayed wound healing commonly observed in diabetic patients.\(^4\) Findings here of increased levels of HbA\(_{1c}\) in diabetic versus control animals coupled with proportional increases observed between levels of HbA\(_{1c}\) and pocket probing depths correlate well with prior work thus supporting previously discussed theoretical hypotheses.

Many have described neutrophil insufficiency as an important component of biological changes within the overall impairment of defense mechanisms in diabetics.\(^25\,\!\!\!^26\) Specifically the transit of neutrophils through vascular walls to inflamed areas is disturbed by arteriosclerosis or microangiopathy. Moreover, since chemotaxis,\(^27\) adhesion\(^26\,\!\!\!^29\) and phagocytosis\(^30\) all require energy, diminished ATP production secondary to impaired neutrophil glycolysis dysfunction (caused by insulin deficiency)\(^31\,\!\!\!^32\) could well lead to compromised defense mechanisms and consequent progression of periodontal disease.

Although various investigators have advanced the longstanding theory proposing a relationship between diabetes mellitus and periodontal diseases, there are still inconsistencies due to differences between reports.\(^33\,\!\!\!^35\) Inconsistencies can be attributed to several factors including human subjects studied, different types of diabetes mellitus, degree of therapeutic control plus the duration of disease and presence or absence of other complications. Experimental animals were therefore used here mainly because of the ease with which variables can be controlled.

Our primary aim in this study was to investigate both chemotaxis and phagocytosis of intraperitoneal macrophages in rats in which diabetes mellitus was experimentally produced by injection of STZ. Further, since ODUS/Odu represent an accepted experimental model for naturally occurring gingivitis, we also studied periodontal changes of induced diabetic rats compared to similar tissues of control animals. At three months following experimental injections, there was a significant decrease in macrophage chemotaxis observed in the diabetic group.
versus control animals (p<0.001). Moreover, both phagocytosis ratios and phagocytosis indices were significantly decreased in a comparable manner (p <0.001).

Reportedly, pocket depth of diabetic experimental animals greater than those of non-diabetic matched controls. However, others report no significant differences between two groups. Still others have reported that although there were no differences between the two groups, many diabetic patients with prolonged duration of disease exhibited pocket depth of 6 mm and greater. Results here are therefore comparable to previously published work in demonstrating significantly greater pocket depths in diabetic versus non-diabetic groups. Also, our data demonstrating positive correlation between the pocket probing depths and blood glucose levels is supportive of the work of Finestone et al who described a positive correlation between plaque index and blood glucose levels in diabetic patients. Our findings demonstrating positive correlation between the pocket probing depths and HbA1C (Y = 39.3 X + 370, r = 0.959, p<0.01) are also consistent with those of previous reports which have shown that patients with poor control of diabetes mellitus experienced more periodontitis than those who were well controlled.

In summary, data here suggest that the inhibition of chemotaxis and phagocytosis seen in macrophages of diabetic animals diminishes the protective effect of leukocytes against nonspecific infection. This could represent at least a partial explanation for observed exacerbation and/or intractability of periodontal diseases in patients afflicted with diabetes mellitus.

In the future, we intend to investigate how or if gingivitis changes with therapy, and whether or not recovery of leukocyte function is possible when diabetic blood glucose levels are controlled by insulin administration.

The authors wish to express their sincere thanks to Dr. K Ogata for his skilful technical assistance, and members of the Department of Pharmacology for their kind advice and help in making this study.

This research was performed with the help of the Experimental Animal Facilities of the Institute of Dental Research, Osaka Dental University.

This study was presented at the 40th Annual Meeting of the Japanese Society of Periodontology, April 24, 1997, Koriyama, Japan.

REFERENCES


