Immunohistochemical study of the periodontal tissues of model rats with type 2 diabetes mellitus

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We investigated the relation between periodontal tissue and type 2 diabetes mellitus and attempted to clarify the distribution of osteoclasts, macrophages, collagens and blood capillaries in periodontal tissue with the diabetes. We immunohistochemically examined CD68-positive cells, type 3 and type 4 collagens, and enzyme histochemically examined TRAP-positive cells in the periodontal tissues of the Goto-Kakizaki (GK) rat, which is a type 2 diabetes model rat. Wistar rats were used as controls.

There were many macrophages and osteoclasts in the periodontal tissue of the diabetes mellitus rats at 2 months of age (DM-2M), whereas localization of type 3 collagen was the same as in the controls. There was an increased tendency for localization of type 4 collagen in the experimental animals compared DM-2M with the controls (NDM-2M). These results suggest that diabetes plays an important role as a risk factor for periodontitis. (J Osaka Dent Univ 2009; 43: 93–102)

Key words: Diabetes mellitus; Periodontal tissues; Immunohistological staining; GK rat

INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia, glycosuria, water and electrolyte loss and weight reduction. These signs are induced by an absolute and relative deficiency of insulin in Langerhans islands of the pancreas and insulin resistance in the target organ. There are primarily two types of the disease, type 1 and type 2. Type 2 diabetes results from a deficiency of insulin secretion from the \( \beta \) cells or from insulin resistance, while type 1 diabetes is an autoimmune disease or results from tissue destruction of the pancreas caused by tumors or inflammation. Most diabetes in adults is this type 2. Hyperglycemia causes considerable complications in both types. Complications include retinopathy and neuropathy, which are classified as microangiopathy, and strokes, myocardial infarctions and angina pectoris, which are classified as macroangiopathy.\(^1\)

Obesity, hypertension, hyperlipemia and hyperglycemia are factors of metabolic syndrome.\(^2\) Metabolic syndrome associated with lack of exercise, smoking, alcohol consumption and stress, increase the risk of arteriosclerosis. According to the 2006 Health and Nutrition Survey of the Ministry of Health, Labour and Welfare, diabetes is thought to afflict about 8,200,000 people in Japan, while another 10,500,000 are at risk for the disease, for a total of 18,700,000.\(^3\) About 95% of all diabetes patients have the type 2 disease. The World Health Organization reports that world wide type 2 diabetes afflicted 135 million people in 1995, and this number will increase to 217 million in 2005 and reach 366 million in 2030.\(^4\)

Diabetes is a risk factor for periodontitis, which is considered the sixth most common complication of the disease.\(^5\) Epidemiological research indicates that the incidence and progression of periodontitis is greater with DM than without.\(^6,7\) It has been reported that the absorption of alveolar bone is increased when blood glucose control is poor.\(^8\) It has
been pointed out that bone metabolic abnormalities induced by diabetes include osteopenia, and it has been suggested that there is a relation between systemic bone metabolic disorders like osteoporosis and alveolar bone resorption in periodontitis. However, it is not clear how DM affects periodontal tissues that do not have periodontitis. We examined immunohistochemically the expression of CD68, type 3 collagen, and type 4 collagen, and examined enzyme histochemically tartrate-resistant acid phosphatase (TRAP) activity in the periodontal tissue of the Goto-Kakizaki (GK) rat, which is a model for type 2 diabetes.

MATERIALS AND METHODS

Animals
We used five, 2-month-old GK rats (DM-2M), and five, 5-month-old GK rats (DM-5M) as the experimental animals. Five, 2-month-old Wistar rats (NDM-2M) and five, 5-month-old Wistar rats (NDM-5M) were used as controls. All animals were from Shimizu Experimental Materials, Kyoto, Japan. This study was approved by the Osaka Dental University Animal Research Committee (approval number 08–02036) and performed in accordance with the guidelines related to animal experiments.

Methods
After fasting for 20 hours, the animals were weighed under isoflurane inhalation anesthesia, and blood was collected from the tail vein to measure the fasting blood glucose level using Nipro Freestyle Flash (Nipro Co., Osaka Japan). The rats were then euthanized by pentobarbital sodium (Nembutal®; Dainippon Sumitomo Pharma Co., Osaka, Japan).

Tissue preparation
After euthanasia, the upper right side of the skull was immersion-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer. The maxillary right first molar regions were resected and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer at 4°C overnight. The specimens were then decalcified with 10% EDTA-2Na and -4Na solutions for about two months. The maxillary right first molar regions were then embedded in paraffin by conventional methods. Serial sections of 5 μm were made in the mesiodistal direction, placed on MAS coat slide® (Matsumani Glass Co., Osaka, Japan), and dried at about 50°C overnight.

Hematoxylin-eosin stain
One set of five sections each were made for the diabetes group and the non-diabetes group. All sections were stained with hematoxylin-eosin.

Enzyme histochemical staining for TRAP
Paraffin-embedded sections were deparaffinized, rehydrated in distilled water, immersed in 0.1 M tris chloride buffer solution at pH 9.0 for 18 hours at 20°C, washed in 0.1 M phosphate buffer solution at pH 7.2, and immersed in 0.1 M citric acid buffer solution at pH 5.2 for 6 hours at 20°C for acid phosphatase reactivation. The sections were then washed in 0.1 M phosphate buffer at pH 7.2 three times each and reacted at 37°C for 30 min with TRAP solution composed of 4 mg naphthol AS-BI phosphate, 0.4 mL of N,N-dimethylformamide, 25 mL of 0.2 M acetic acid buffer solution at pH 5.0, 35 mg fast red violet LB and 187.5 mg (50 mM) of 10% magnesium chloride 60 μL and L(+)-tartaric acid 187.5 mg (50 mM) adjusted to pH 5.0 by 1 N NaOH. The sections were then washed, counterstained in hematoxylin, embedded and observed by light microscopy.

Immunohistochemical staining for CD68
Dепaraffinized sections were then washed in distilled water and antigen-retrieved in 0.05% trypsin at room temperature for 10 min. They were then immersed in 3% H2O2 for 5 min to block endogenous peroxidase. The sections were incubated with a 1:800 dilution of anti-CD68 monoclonal antibody (AbD Serotec, Oxford, UK) at room temperature for 60 min. The sections were then reacted with Histofine Simple Stain Rat MAX-PO (M) (Nichirei Bioscience, Tokyo, Japan) at room temperature for 30 min and visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (DAKO, Glostrup, Den-
mark) at room temperature for 5 min. They were then counterstained in hematoxylin, embedded and observed by light microscopy.

**Immunohistochemical staining for collagen types 3 and 4**

Deparaffinized sections were washed in distilled water, antigen-retrieved in 0.4% pepsin at 37°C for 10 min, and immersed in 3% H₂O₂ for 5 min to block endogenous peroxidase. They were then incubated with a 1:1,000 dilution of anti-type 3 collagen antibody or with a 1:1,000 dilution of anti-type 4 collagen antibody (both from Novotec, Saint Martin La Garenne, France) at room temperature for 60 min. The sections were then reacted with peroxidase conjugated Envision polymer at room temperature for 30 min and visualized with DAB at room temperature for 5 min. The sections were then counterstained with hematoxylin, embedded and observed by light microscopy.

**RESULTS**

**Weight and blood glucose level**

The GK rats of the diabetes group had a lower average weight than the Wistar rats of the control group (Table 1), and a higher average blood glucose level (Table 2).

**Histopathological findings**

In DM-2M, although we observed neutrophil infiltration and capillaries adjacent to the oral sulcular epithelium, cementum hyperplasia of the root apex was rarely seen (Figs. 1A–1 and 1A–2). There were more capillaries than in the NDM-2M (Fig. 1B–1). A difference was not observed in the alveolar bone between DM-2M and NDM-2M (Fig. 1B–2). In DM-5M, we observed neutrophil infiltration and the capillaries near the oral sulcular epithelium (Fig. 1C–1). However, there was no difference in the alveolar bone or cementum between DM-5M and NDM-5M (Figs. 1C–2, 1D–1 and 1D–2).

**Enzyme histochemical findings for TRAP**

In DM-2M, many TRAP positive multinucleated giant cells and osteoclasts were observed from the alveolar bone around the root apex to the alveolar crest. A few TRAP-positive multinucleated giant cells were observed on the surface of the alveolar bone in the mid portion root and many TRAP-positive multinucleated giant cells were observed in the alveolar bone around the root apex (Figs. 2A–1 and 2A–2). Many TRAP-positive cells were observed in DM-2M compared with NDM-2M (Fig. 2B–1 and 2B–2). In DM-5M, TRAP positive cells were observed on the alveolar bone surface from near the apex to the central part of the root, and osteoclasts were observed (Figs. 2C–1 and 2C–2). In particular, they were observed near the root apex and there was a little difference between NDM-5M and DM-5M (Figs. 2D–1 and 2D–2).

**Immunohistochemical findings for CD68**

In DM-2M, CD68 revealed a large number of multinucleated giant cells from the alveolar bone around the root apex to the alveolar crest, and multinucleated cells with CD68-positivity were observed near the oral sulcular epithelium (Figs. 3A–1 and 3A–2). As compared with NDM-2M, many multinucleated giant cells with CD68-positivity were observed from the alveolar bone near the root apex to the alveolar crest (Figs. 3B–1 and 3B–2). In DM-5M, the multinucleated giant cells with CD68-positivity were observed from the alveolar crest to the alveolar bone near the root apex. They were generally observed near the root apex in particular. They were ob-

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**Table 1**  Body weight of the diabetic and control groups

<table>
<thead>
<tr>
<th>Age (mo)</th>
<th>Diabetic group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>179.38 ± 14.00</td>
<td>240.00 ± 9.35</td>
</tr>
<tr>
<td>5</td>
<td>362.50 ± 6.55</td>
<td>413.00 ± 10.95</td>
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</tbody>
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Mean±SD, p<0.01. (g)

**Table 2**  Blood glucose level of the diabetic and control groups

<table>
<thead>
<tr>
<th>Age (mo)</th>
<th>Diabetic group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>128.88 ± 16.86</td>
<td>82.56 ± 18.48</td>
</tr>
<tr>
<td>5</td>
<td>215.75 ± 24.71</td>
<td>88.6 ± 3.05</td>
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</tbody>
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Mean±SD, p<0.01. (mg/dL)
Fig. 1  Hematoxylin-eosin staining of periodontal tissue.
1A–1, 1A–2 : 2-month-old GK rats (DM-2M),  
1B–1, 1B–2 : 2-month-old Wistar rats (NDM-2M),  
1C–1, 1C–2 : 5-month-old GK rats (DM-5M),  
1D–1, 1D–2 : 5-month-old Wistar rats (NDM-5M),  
1A–1, 1B–1, 1C–1, 1D–1 : Coronal portion of periodontal tissue,  
1A–2, 1B–2, 1C–2, 1D–2 : Apical portion of periodontal tissue.
Enzyme histochemical staining by TRAP of periodontal tissue.
2A-1, 2A-2: 2-month-old GK rats (DM-2M),
2B-1, 2B-2: 2-month-old Wistar rats (NDM-2M),
2C-1, 2C-2: 5-month-old GK rats (DM-5M),
2D-1, 2D-2: 5-month-old Wistar rats (NDM-5M),
2A-1, 2B-1, 2C-1, 2D-1: Coronal portion of periodontal tissue,
2A-2, 2B-2, 2C-2, 2D-2: Apical portion of periodontal tissue.

served near the oral sulcular epithelium (Figs. 3C-1 and 3C-2). They were a few more from the alveolar bone to the alveolar crest than was the case with NDM-5M (Figs. 3D-1 and 3D-2).

Immunohistochemical findings for collagen type 3
In DM-2M and DM-5M, type 3 collagen was localized throughout the periodontal tissue (Figs. 4A-1, 4A-2, 4C-1 and 4C-2). There was virtually no difference in location between NDM-2M and NDM-5M (Figs. 4B-1, 4B-2, 4D-1 and 4D-2).

Immunohistochemical findings for collagen type 4
In DM-2M, type 4 collagen was localized in the epi-
Fig. 3  Immunohistochemical staining by CD68 of periodontal tissue.
3A-1, 3A-2: 2-month-old GK rats (DM-2M),
3B-1, 3B-2: 2-month-old Wistar rats (NDM-2M),
3C-1, 3C-2: 5-month-old GK rats (DM-5M),
3D-1, 3D-2: 5-month-old Wistar rats (NDM-5M),
3A-1, 3B-1, 3C-1, 3D-1: Coronal portion of periodontal tissue,
3A-2, 3B-2, 3C-2, 3D-2: Apical portion of periodontal tissue.
Fig. 4  Immunohistochemical staining of collagen 3 in periodontal tissue.
4A-1, 4A-2: 2-month-old GK rats (DM-2M),
4B-1, 4B-2: 2-month-old Wistar rats (NDM-2M),
4C-1, 4C-2: 5-month-old GK rats (DM-5M),
4D-1, 4D-2: 5-month-old Wistar rats (NDM-5M),
4A-1, 4B-1, 4C-1, 4D-1: Coronal portion of periodontal tissue,
4A-2, 4B-2, 4C-2, 4D-2: Apical portion of periodontal tissue.
Fig. 5  Immunohistochemical staining of collagen 4 in periodontal tissue.

5A-1, 5A-2: 2-month-old GK rats (DM-2M),
5B-1, 5B-2: 2-month-old Wistar rats (NDM-2M),
5C-1, 5C-2: 5-month-old GK rats (DM-5M),
5D-1, 5D-2: 5-month-old Wistar rats (NDM-5M),
5A-1, 5B-1, 5C-1, 5D-1: Coronal portion of periodontal tissue,
5A-2, 5B-2, 5C-2, 5D-2: Apical portion of periodontal tissue.
the epithelial basement membrane and the capillary basement membrane (Figs. 5A–1 and 5A–2). It was more common than in NDM-2M of the control (Figs. 5B–1 and 5B–2). In DM-5M, type 4 collagen antibody was localized in the epithelial basement membrane and the blood capillary basement membrane (Figs. 5C–1 and 5C–2). There was little difference compared with NDM-5M (Figs. 5D–1 and 5D–2).

**DISCUSSION**

Since Albright\(^\text{13}\) reported osteopenia in diabetes in 1948, many epidemiologic studies have been performed. Although it is well known that osteopenia is present in type 1 diabetes, osteopenia is not clearly observed in type 2 diabetes. In recent years, bone metabolism in type 2 diabetes has been noted to be a low turnover type of osteoporosis. Low bone metabolic turnover, which means low turnover of bone formation and resorption, depends primarily on the hypoparathyroidism and the low turnover of osteoblasts.\(^\text{14}\) Osteoclasts derived from blood stem cells are the only cells that destroy and absorb bone tissues. There were more macrophages and osteoclasts in DM-2M than in NDM-2M. Although many osteoclasts were observed, there was very little resorption of alveolar bone in the streptozotocin (type 1 diabetes model) mice.\(^\text{15}\) In addition, high turnover type bone resorption is found in DM of the early stage or DM that is poorly controlled.\(^\text{14}\) This might be similar to the results we found in DM-2M, which is in the early stage of DM. Moreover, it has been noted that in a coculture of mouse bone marrow cells and stromal cells, osteoclast formation is promoted under high glucose concentration.\(^\text{16}\) Thus, DM is associated with bone metabolism and has the potential to promote osteoporosis.

Collagen is the main component of periodontal tissue as well as the organic matrix of alveolar bone. Collagen metabolism influences wound healing, and exacerbation and progression of periodontitis. DM inhibits the healing process of periodontitis in the gingival tissue of diabetic rats\(^\text{17}\) by activating collagenolytic enzymes. We found no difference in the localization of type 3 collagen in the periodontal tissue between the DM and NDN groups. However, there was more type 4 collagen in DM-2M than in NDM-2M. Type 3 collagen, which has reticular fibers and exists in the gingiva, cementum and periodontal ligament, is increased in the early stages of the wound-healing. It is replaced by type 1 collagen as healing progresses. Thus, type 3 collagen is strongly associated with the promotion of wound healing. Because type 4 collagen exists in the basement membrane of epithelium and blood vessels, studying collagen is useful for understanding the basement membrane of epithelium and blood vessels.

We are also indebted to the staff and graduate students of the Departments of Oral Pathology and Periodontology. We wish to express our sincere gratitude to the late Professor Hisao Inai of the Department of Periodontology who guided us from the beginning of this research. We also wish to thank Mr. Hideaki Hori who helped us with the use of morphological research and photograph-processing facilities, and the staff in the animal facilities.

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REFERENCES