Morphological Study of the Palatal Gingiva of the Maxillary First Molar in the Type 2 Diabetes Mellitus Model Rat

By

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Summary: We studied morphological changes at the maxillary first molar in a model rat for type 2 spontaneous diabetes mellitus (DM), the Goto-Kazizaki (GK) rat, vs. the normal 8-week-old Wistar rat. Serial frontal sections of the gingiva of the maxilla with the bone were prepared from the rats. Image analyses, performed on light micrographs of the hematoxylin-eosin stained specimens, allowed comparison of the thickness of the keratinized, granular, prickle, and basal layers. In addition, the cell population of the granular and prickle layers and the cross-sectional area of the connective tissue beneath the mucosal epithelium were examined. The thickness of the capillary of the maxillary first molar was determined by image analysis of scanning electron micrographs of microvascular corrosion cast specimens. We found that the thickness of the keratinized, granular, and prickle layers was significantly higher in the DM vs. normal group, as were the cell population of the granular and prickle layers. In contrast, the cross-sectional area of the connective tissue beneath the mucosal epithelium, and the thickness of the capillary were significantly lower in the DM vs. normal sections. Therefore, we consider that the DM-associated hyperglycemia causes hypertrophy of the mucosal epithelium, atrophy of the connective tissue beneath the mucosal epithelium, and microangiopathy of the capillary of the palatal gingiva of the maxillary first molar in the GK rat.
Heparin Injection 5000®
Mochida Pharmaceutical Co., Ltd., Tokyo, Japan), and after 30 minutes, they were euthanized with an intraperitoneal injection of excess sodium pentobarbital (Nembutal®, Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan). The thorax was opened, and blood was collected from the left ventricle with a syringe (5-ml Terumo Syringe®, TERUMO, Tokyo, Japan) for measurements of fasting blood glucose and hemoglobin A1c levels. After cannulation was performed from the left ventricle to the ascending aorta, isotonic saline was infused into the ascending aorta. Specimens were collected from all rats after withdrawing blood from the right atrial auricle.

Measurement of fasting blood glucose levels
Fasting blood glucose levels were measured in rat serum samples with the HK-G-6-PDH (Quick auto neo GLU-HK, Sino Test Co., Ltd., Tokyo, Japan) according to instructions of the manufacturer. Sera were obtained by centrifugal separation of the collected blood samples.

Measurement of hemoglobin A1c levels
Hemoglobin A1c levels were determined by use of the latex agglutination (RAPIDIA®, AUTO HbA1c-L, FUJIREBIO INC. Tokyo, Japan) according to instructions of the manufacturer. Sera were obtained by centrifugal separation of the collected blood samples.

Preparation of specimens
1. Hematoxylin-eosin stained specimens
Specimens from 3 rats of each group were used to prepare sections of the palatal gingiva of the maxillary first molar. A 4% (w/v) paraformaldehyde solution (Formaldehyde Solution®, Kishida Chemical Co., Ltd., Osaka, Japan) was infused into the ascending aorta. The gingivae of the maxilla with the bone were removed and then fixed by soaking in 4% paraformaldehyde at 4 °C for 24 hours. These specimens were decalcified in 10% (w/v) ethylene diamine tetraacetic acid disodium salt (EDTA) solution for 10 days using a microwave rapid sample processor (ML-77, Azumaya Corp., Tokyo, Japan). These specimens were frozen after washing. Serial frontal sections of 20-μm thickness were sliced distally from the medial surface of the maxillary first molar teeth with a cryostat (HM500-OM, Carl Zeiss Japan, Tokyo, Japan). The sections were stained with a hematoxylin-eosin solution. Digital images of the stained sections were acquired using a light microscope equipped with a digital camera (BZ-9000, Keyence Corporation, Osaka, Japan).

2. Microvascular corrosion cast specimens
Three rats from each group were used to investigate the microvascular architecture of the palatal gingiva of the maxillary first molar. To remove hydroquinone (a polymerization inhibitor) from methylmethacrylate (Acrylic ester M®, MMA, Mitsubishi Rayon Co., Ltd., Tokyo, Japan), the liquid monomer was purified by distillation. One part by weight of polymethyl methacrylate (solid polymer; Sigma-Aldrich Japan K.K., Tokyo, Japan) was added to 9 parts of purified liquid monomer, to prepare the low viscosity acrylic resin. High viscosity acrylic resin was prepared from a 3:7 mixture by weight of polymer and monomer, respectively. To these mixtures, 0.5% polymerization promoter (benzoyl peroxide, Kishida Chemical Co., Ltd.), 5% plasticizer (di-n-butyl phthalate, Kishida Chemical Co., Ltd.), pigment (Cromophial Red, Ciba Japan K.K., Tokyo, Japan), and 0.5% polymerization initiator (N,N-dimethylaniline, Kishida Chemical Co., Ltd.) were added to initiate polymerization. First, the low viscosity acrylic resin (45 mL, 5 mL/minute) was injected into rats from the ascending aorta using a precise syringe pump (KDS200, Muromachi Kikai Co., Ltd., Tokyo, Japan), after which the high viscosity acrylic resin (5 mL, 1 mL/minute) was injected from the same aorta and pump. The injected animals were allowed to polymerize at 40 °C for 24 hours in a water bus (Thermo Regulator, CTR-320, Iwaki Co., Ltd, Tokyo, Japan). Specimens were collected from the polymerized animals, and then, the soft tissues were removed by soaking the specimens in a 12% low-salt sodium hypochlorite solution (Hypochlorite soda®, Fukae Trading Company Co., Ltd., Osaka, Japan) at 40 °C for 24 hours. The specimens were then washed in an ultrasonic cleaner (UT-105HS, Sharp Corporation, Osaka, Japan) with running hot water at 40 °C. Each specimen was air-dried, mounted on a metal stage with silver paste (Dotite®, Fujikura Kasei Co., Tochigi, Japan), and coated with gold using an ion-sputtering coating device (JFC-1500, JEOL, Tokyo, Japan).

Digital images of the microvascular architecture of the palatal gingiva of the maxillary first molar were obtained by scanning electron microscope (JSM-5500, JEOL) at an acceleration voltage of 5 kV and a working distance of 40 mm.

Image and statistical analyses
The images of hematoxylin-eosin stained sections were analyzed to determine tissue layer thickness and cell population using measurement software (BZ-2 Analyzer, Keyence Corporation). In the digital images of the microvascular corrosion cast specimens, the thickness of the capillaries was measured with Image-Pro measurement software (Image-Pro Plus® 5.0J, Nippon Roper, Tokyo, Japan). The measurements were also calculated and represented as mean values ± standard deviations, and Student’s t test was used to test for statistical significance of differences between the 2 groups. Differences were considered significant when p < 0.01.

1. Thickness of mucosal epithelium
In the digital images, a standard line (line S) that passed the cement-enamel junctions (CEJ) on the palate side of
The right and left maxillary first molars was drawn. Then, a perpendicular line (P) was drawn from the top of the gingiva to line S, and it was divided equally into 3 parts. For the sake of convenience, the upper part (U) was named the “gingival top”, middle part (M) was called the “gingival center”, and the lower part (L) was referred to as the “gingival bottom” (Fig. 1a). Five epithelial processes were selected randomly from the 3 parts. The most inferior point of the epithelial process was named “z”. A perpendicular line (line u) has been drawn from “z” to the tangent line on the surface of the maxillary gingival mucosa. v: intersection of line u and surface of keratinized layer, w: intersection of line u and deepest point of keratinized layer, x: intersection of line u and deepest point of the granular layer, y: intersection of line u and deepest point of prickle layer.

Fig. 1. Schematic illustration in the palatal gingiva of the maxillary first molar.

a. The measurement definition of the thickness of the mucosal epithelium and the cross-sectional area of the connective tissue beneath the mucosal epithelium (Co) in the palatal gingiva of the maxillary first molar (lower magnification). CEJ: cement-enamel junction, D: dentine, E: decalcified enamel, P: perpendicular line, S: standard line, U: upper part, M: middle part, L: lower part.
b. The measurement definition of the thickness of the keratinized (k), the granular (g), the prickle (p), and the basal (b) layers. Higher magnification of the black flame in 1a. The most inferior point of the epithelial process is named “z”. A perpendicular line (line u) has been drawn from “z” to the tangent line on the surface of the maxillary gingival mucosa.

1) Thickness of keratinized layer
The intersection of line u and the deepest point of the keratinized layer was named “w”. The length from “v” to “w” was defined as the thickness of the keratinized layer (k).

2) Thickness of granular layer
The intersection of line u and the deepest point of the granular layer was named “x”. The length from “w” to “x” was defined as thickness of the granular layer (g).
3) Thickness of prickle layer
The intersection of line u and the deepest point of the prickle layer was named “y”. The length from “x” to “y” was defined as thickness of the prickle layer (p).

4) Thickness of basal layer
The length from “y” to “z” was defined as thickness of the basal layer (b).

2. Cell population of granular and prickle layers
The cell numbers of the granular and prickle layers on line u were counted.

3. Cross-sectional area of connective tissue beneath mucosal epithelium
The area of the connective tissue above line S was defined as the cross-sectional area of the connective tissue beneath the mucosal epithelium. Refer to the shaded area (Co) in Fig. 1a to visualize this measurement.

4. Thickness of capillary
The diameter of a capillary of the first maxillary molar was defined as the thickness of a capillary in the superior margin of a capillary network at the superior edge of the palatal gingiva.

Results

Fasting blood glucose levels
The fasting glucose levels were found to be significantly higher in the DM group than in the normal group ($p < 0.01$), as shown in Fig. 2 and Table 1. The levels were 118.44 ± 21.32 mg/dL in the normal group and 181.44 ± 20.33 mg/dL in the DM group.

Table 1. Comparison of fasting blood glucose levels and hemoglobin A$_{1c}$ in normal (N) and diabetes mellitus (DM) groups ($*: p < 0.01$).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting blood glucose levels</td>
<td>N</td>
</tr>
<tr>
<td>DM</td>
<td>181.44 ± 20.33 mg/dL*</td>
</tr>
<tr>
<td>Hemoglobin A$_{1c}$ levels</td>
<td>N</td>
</tr>
<tr>
<td>DM</td>
<td>4.78 ± 0.20%*</td>
</tr>
</tbody>
</table>

Hemoglobin A$_{1c}$ levels
The hemoglobin A$_{1c}$ levels were found to be significantly higher in the DM group than in the normal group ($p < 0.01$), and these data were shown in Fig. 3 and Table 1. The levels were 4.22 ± 0.16% in the normal group and 4.78 ± 0.20% in the DM group.

Findings of specimens

1. Hematoxylin-eosin stained specimens
In both the normal and DM groups, the mucosal epithelium of the palatal gingiva of the maxillary first molar appeared as a keratinized stratified squamous epithelium, distinct from the various other layers (e.g., keratinized, granular, prickle, and basal). The proper lamina of the dense connective tissue was observed beneath the mucosal epithelium. In the keratinized layer, the anucleate keratinocytes (stained only with eosin) could be discerned. The epithelial surface of the keratinized layer presented a parallel pattern in the normal group, and an undulated pattern in the DM group. In the granular layer, hematoxylin-stained granular cells containing keratohyaline granules were visible. Cells of the prickle layer were also observed.
In the basal layer, which was a single layer, the columnar cells could be seen queued up like paving stones (Figs. 4a and b).

2. Microvascular corrosion cast specimens

In the normal group, the shapes of the capillary networks in the gingival top (U) of the palatal gingiva were rectangular (shown by the asterisks in Fig. 5b), and the capillary of the superior margin ran in a straight pattern (shown by the arrow heads in Fig. 5b). However, the shapes of capillary networks in the gingival top (U) of the palatal gingiva were oval in specimens from the DM group (shown by the triangles in Fig. 5d), and the capillary of the superior margin ran in an undulated pattern (shown by the arrows in Fig. 5d).

Image and statistical analyses

1. Thickness of mucosal epithelium

The thicknesses of the mucosal epithelium were 110.21 ± 43.89 μm in the normal group and 171.85 ± 57.67 μm in DM groups, and this difference was significant \( (p < 0.01; \text{Fig. 6 and Table 2}) \).

1) Thickness of keratinized layer

The thicknesses of the 3 parts (U, M, and L) of the keratinized layer were shown in Table 3. The corresponding values were consistently and significantly higher in specimens of the DM group than in the normal group \( (p < 0.01; \text{Figs. 7–9 and Table 3}) \). The thicknesses of the keratinized layer were 25.68 ± 7.36 μm in the normal group and...
These differences were significant ($p<0.01$).

2) Thickness of granular layer

The thicknesses of the 3 parts (U, M, and L) of the granular layer were shown in Table 3. These values in each part were significantly higher in the DM group than in the normal group ($p<0.01$; Figs. 7–9 and Table 3). The thicknesses of the granular layer were $13.16 ± 4.64 \mu m$ in the normal group and $31.03 ± 10.47 \mu m$ in the DM group (Fig. 10 and Table 3). These differences were significant ($p<0.01$).

3) Thickness of prickle layer

The thicknesses values of the prickle layer (divided into the U, M, and L parts) were shown in Table 3. These
values in each part were significantly higher in the DM group than in the normal group (p < 0.01; refer to Figs. 7–9 and Table 3). The thicknesses of the prickle layer were 60.56 ± 31.57 μm in the normal group, which was significantly lower than that in the DM group (81.38 ± 33.86 μm) (p < 0.01; Fig. 10 and Table 3).

4) Thickness of basal layer
The thicknesses of the 3 parts (U, M, and L) of the basal layer were shown in Table 3. There was no significant difference between any of the 3 parts of the 2 groups (p > 0.01; Figs. 7–9 and Table 3). The thicknesses of the basal layer in the normal group (10.82 ± 3.56 μm) were
2. Cell population of granular and prickle layers

1) Cell population of granular layer
   The cell populations of the granular layer were 2.31 ± 0.51 in the normal group and 3.60 ± 0.81 in the DM group. These values were significantly higher in the DM group than in the normal group (p < 0.01; Fig. 11 and Table 2).

2) Cell population of prickle layer
   The cell populations of the prickle layer were 6.31 ± 2.09 in the normal group and 8.73 ± 2.76 in the DM group. These values were significantly higher in the DM group than in the normal group (p < 0.01; Fig. 12 and Table 2).

3. Cross-sectional area of connective tissue beneath mucosal epithelium
   The cross-sectional areas of the connective tissue beneath the mucosal epithelium were 93899.74 ± 30784.40 μm² in the normal group and 43204.19 ± 12725.23 μm² in the DM group. These values were significantly lower in the DM group than in the normal group (p < 0.01). These results were shown in Fig. 13 and Table 2.

4. Thickness of capillary
   The thicknesses of the capillaries (Fig. 14 and Table 2) were 14.99 ± 1.59 μm in the normal group and 8.88 ± 2.74 μm in the DM group. These values were significantly lower in the DM group than in the normal group (p < 0.01).
**Discussion**

*Experimental animals*

In research of DM, there were several animal models available. Type 1 DM may be studied in rats that were administered streptozotocin (STZ). In this model, STZ destroyed the β cells of the pancreas islet, and this results in the induction of type 1 DM. However, STZ might also influence the kidney and liver\(^7\),\(^8\). It was of note that these rats showed extremely high fasting blood glucose levels, on the order of 421–573 mg/dL\(^9\),\(^10\). A second model that was well known was the GK rat as model for type 2 DM, and in these animals the fasting blood glucose levels were much lower (181.44 ± 20.33 mg/dL). Among adult Japanese with type 2 DM, or borderline DM, a low fasting blood glucose level was characteristic. For this reason, the experimental animal that we chose to study was the type 2 spontaneous DM model GK rat.

**Gingival mucosa**

1. **Mucosal epithelium**

   The mucosal epithelium was about 1.6-times thicker in the DM group than in the normal group. Each of the constituent layers was considered below.

   1) Keratinized layer

   The keratinized layer was about 1.9-times thicker in the DM group than in the normal group. This finding supported *in vitro* studies that demonstrate increased keratin levels, and larger and flattened cell morphology, in keratinocytes that were grown in the presence of high glucose concentrations\(^11\). Therefore, one reason the keratinized layer in the GK rats became thicker might be the increased keratin content of the keratinocytes.

   2) Granular layer

   The granular layer was about 2.3-times thicker and the cell population was about 1.6-times higher in the DM group than in the normal group. This observation was consistent with the known increased proliferation of oral epithelial cells *in vitro* in the presence of high glucose concentrations\(^12\).

   3) Prickle layer

   Both the thickness and cell population of the prickle layer were about 1.6-times greater in the DM group than in the normal group. Because it was known that proliferation of oral epithelial cells is promoted *in vitro* by high glucose\(^12\), this may be a reason for the thicker prickle layer in the GK rats.

   4) Basal layer

   The basal layer in both the groups was visible as a single layer, and there was no significant difference in thickness or morphology of the layer between the 2 groups.

5) **Comparison of mucosal epithelium of lingual gingiva of mandibular first molar**

   In this study, the mucosal epithelium of the palatal gingiva of the maxillary first molar was thicker and the cell populations of the granular and prickle layers were higher in GK than in normal Wistar rats. However, it had been reported that the mucosal epithelium of the lingual gingiva of the mandibular first molar was thinner in the GK than in normal rats\(^13\). This observation was of interest, as it suggested that the hyperglycemic effect was different in the mucosal epithelium of the palatal vs. lingual gingiva of the corresponding first molars.

2. **Connective tissue beneath mucosal epithelium**

   The cross-sectional area of the connective tissue beneath the mucosal epithelium was about 0.5-times narrower in the DM group than in the normal group. In the GK rats, DM had been reported to cause atrophic changes in the connective tissue papillae of the lingual gingiva of the mandibular first molar\(^2\) and in the connective tissue papilla in the filiform papillae of the lingual dorsum\(^13\). In this connection, it had been observed that GK dermal fibroblast proliferation *in vitro* was suppressed by high glucose\(^14\). Therefore, one explanation for the narrower cross-sectional area in GK rats might be suppression of fibroblast proliferation caused by the hyperglycemia.

3. **Capillary**

   The thickness of capillaries was about 0.5-times thinner in the DM group than in the normal group. Diabetic microangiopathy in the capillary loops in the filiform papillae of the lingual dorsum in GK rats had been reported\(^13\). In this regard, it was of note that the proliferation of vascular endothelial cells was suppressed *in vitro* by high glucose\(^15\). The thinner capillary in GK rats might be due to hyperglycemic suppression of the vascular endothelial cell proliferation that results in the development of a characteristic diabetic microangiopathy.

**Conclusions**

On the basis of the comparison between the mucosal epithelium, connective tissue beneath the mucosal epithelium, and capillary of the palatal gingiva of the maxillary first molar in normal Wistar and diabetic GK rats, the following conclusions relevant to this animal model for DM disease were drawn:

1. Hyperglycemia caused a hypertrophy in the mucosal epithelium.
2. Hyperglycemia resulted in atrophy of the connective tissue beneath the mucosal epithelium.
3. Diabetic microangiopathy in the capillary resulted from hyperglycemia.
4. Hyperglycemic effect was different in the mucosal epithelium of the palatal vs. lingual gingiva.
Acknowledgments

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