Morphological Study of the Connective Tissue Papillae and the Capillary Loops on the Lingual Dorsum in the Type 2 Diabetes Mellitus Model Rats

By

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Summary: We performed a morphological study in type 2 diabetes mellitus (DM) model rats by focusing on the connective tissue papillae (CTPe) and capillary loops (CLs) of filiform papillae in the lingual dorsum in the type 2 DM model rats (GK rats). The normal group comprised nine 8-week-old male Wistar rats. The DM group comprised nine 8-week-old male GK rats. Image analysis of light microscopic specimens in CTPe was performed (height and cross-sectional area). Image analysis of scanning electron microscopic specimens (acrylic plastic injection method) in CLs was performed (number, thickness, height, interval between the tops of the two CLs, and crossing ratio between ascending and descending crura (CRC)). We compared these values between both groups (Student’s t test). The former analysis revealed that the height and cross-sectional area of CTPe were smaller in the DM group than in the normal group. The latter analysis revealed that the thickness, height, and CRC of the CLs were smaller in the DM group than in the normal group. However, no significant differences were detected in the number and interval of CLs between both groups. Therefore, we concluded that DM caused regressive change in CTPe and CLs.

Materials and methods

1. Materials

A total of 18 experimental animals (Shimizu Laboratory Supplies Co. Kyoto, Japan) were used in this study. Nine male Wistar rats (8 weeks old, body weight 200–220 g) were used as the normal group and nine male GK rats (8 weeks old, body weight 190–210 g), as the DM group. Three rats from each group were used for the preparations of the light microscope, and also six rats for those of the scanning electron microscope.

This animal study was approved by the Osaka Dental University Animal Research Committee (approval number 07-02015) and performed in accordance with the guidelines related to animal experiments.

2. Methods

The rats were fasted for 20 hours, following which their body weights were measured. Under isoflurane inhalation anesthesia (Forane®, Abbott Japan Co., Ltd., Tokyo, Japan), the rats were intraperitoneally injected with heparin sodium (1,000 units; Novo Heparin Injec-
tion 5000®, Mochida Pharmaceutical Co., Ltd., Tokyo, Japan). 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. The blood was collected from the left ventricle, and the fasting blood glucose level was measured by the method described in section 1). Physiological saline was then infused into the ascending aorta, and specimens of all rats were prepared after withdrawing blood from the right atrial auricle by using the method described in section 2).

1) **Measurement of fasting blood glucose levels**

Serum was obtained after the centrifugal separation of blood and used to measure the fasting blood glucose level using HK-G-6-PDH reagent (Quick auto neo GLU-HK, Sino Test Co., Ltd., Tokyo, Japan). In this study, the DM group was composed of nine GK rats with a fasting blood glucose level of ≥ 150 mg/dL, according to the report of Goto and Kakizaki.

2) **Preparation of specimens**

(1) **Preparation of light microscopic specimens**

Three rats from each group were used to investigate the histological structure of CTPe. The tongues were removed and fixed by soaking in 4% paraformaldehyde solution at 4°C for 24 hours. Serial sagittal sections (30 μm in thickness) were cut laterally from the sagittal plane (0.5 mm outside the lingual median plane) using a cryostat (HM500-OM, Carl Zeiss Japan, Tokyo, Japan). These sections were stained with hematoxylin-eosin.

(2) **Preparation of scanning electron microscopic specimens**

Six rats from each group were used to investigate the microvascular architecture of CLs in the CTPe. Acrylic plastic resin was injected via the ascending aorta by using the plastic injection method. All the tongues were removed after hardening of the resin. The microvascular corrosion casts of the lingual dorsum and those of the sagittal section were prepared according to the methods described below.

1. **Microvascular corrosion casts of the lingual dorsum**

Three rats from each group were used for preparing microvascular corrosion casts of the lingual dorsum. The soft tissues of the specimens were removed by soaking in 10% sodium hydroxide solution. The specimens were then washed with an ultrasonic cleaner (UT-105HS, SHARP Corporation, Osaka, Japan) and with running water. Each specimen, after natural drying, was mounted on a metal stage by using silver paste (DOTITE®, Fujikura Kasei Co., Tochigi, Japan), and coated with gold (4–5 nm in thickness) using an ion-sputtering coating device (JFC-1500, JEOL, Tokyo, Japan). Microvascular corrosion casts of the lingual dorsum were observed at the center of the sagittal line (0.5 mm outside the lingual median plane).

2. **Microvascular corrosion casts of the sagittal section**

Three rats from each group were used for preparing microvascular corrosion casts of the sagittal section. The excised tongues were immediately frozen to observe the microvascular architecture of the sagittal section. The tongues were trimmed medially from the lateral margin to the sagittal plane (0.5 mm outside the lingual median plane) using a cryostat. The microvascular corrosion casts of the sagittal section were prepared using the same method described in the previous section.

3. **Image and statistical analyses**

To observe the CTPe and CLs of the numerous filiform papillae on the lingual dorsum, we equally divided it from the lingual apex to the prominence into the following four zones: apex, anterior, middle, and posterior (Fig. 1). The histological structures of the CTPe were observed, and digital images were acquired using the image input device of the light microscope (AX80 HDTV SYSTEM, Olympus Corporation, Tokyo, Japan) in the sections stained with hematoxylin-eosin. The digital images of the CLs were acquired using the scanning electron microscope.

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**Fig. 1.** Superior view of the lingual dorsum. We equally divide the tongue from the lingual apex to the prominence (P) into the following four zones: ① apex, ② anterior, ③ middle, and ④ posterior. MP; lingual median plane, scale bar = 1 mm.
In the electron microscope at an acceleration voltage of 5 kV and a working distance of 40 mm (JSM-5500, JEOL, Tokyo, Japan) in the microvascular corrosion casts.

Image analyses of the specimens was performed in all the four zones by using an imaging analysis/measurement software (Image Pro Plus 5.0®️, Nippon Roper, Japan) and a personal computer. The average values of all the zones were also calculated and represented as mean ± standard deviation. The Student’s t test was used to test the statistical significance (p < 0.05) between both groups.

1) Image analyses of light microscopic specimens

Five digital images were randomly selected from images of the four zones from each experimental animal. (1) Height of connective tissue papillae

Five CTPe were randomly selected from each digital image. As shown in Fig. 2, line A was drawn between the lowest points of the two adjacent epithelial processes. Further, the perpendicular line (line B) was drawn from the top of the CTPe to line A. The length of line B was measured as the height of the CTPe. (2) Cross-sectional area of connective tissue papillae

Five CTPe were randomly selected from each digital image. As shown in Fig. 2, the area above line A was measured as the cross-sectional area of the CTPe.

2) Image analyses of scanning electron microscopic specimens

The digital images of the above-mentioned zones of five rats were used to examine the CLs in these rats. The CLs of rats (1), (2), (3), and (5) were counted and measured using microvascular corrosion casts of the lingual dorsum (range of 0.3 mm × 0.2 mm), and those of rat (4) using the microvascular corrosion casts of the sagittal section (range of 0.3 mm × 0.2 mm). (1) Number of capillary loops

The number of CLs was counted excluding incomplete CLs such as those in which certain portions of the hairpin loop were not captured in the digital images. (2) Interval between the tops of two capillary loops

We focused on one CL in each of the digital images and randomly selected 5 CLs adjacent to the center of the focused CL. Then, we measured five intervals between the tops of the focused CL and those of the selected CLs. (3) Thickness of capillary loops

Five CLs were randomly selected from each of the digital images, and the thickness of the top of the CLs was measured. (4) Height of capillary loops

We randomly selected five CLs from each digital image and measured their height from the top to the point where the descending crus of the CLs poured into the venous network. (5) Crossing ratio between the ascending and descending crura in capillary loops (CRC)

In all the CLs observed within the range, those that showed no crossing between the ascending and descending crura were defined as Type 1 and those that showed at least one crossing between two crura, as Type 2 (Fig. 3). The number of CLs of each type was counted. We calculated the ratio of the number of Type 2 CLs to the total number of CLs \{(Type 1) + (Type 2)} by using the following formula: \((Type 2)/{(Type 1) + (Type 2)} \times 100\%\).

4. Comparison of the decrease in parameters in the four zones

We measured the height and cross-sectional area of CTPe and the thickness, height, and CRC of CLs in these four zones. Then, we calculated the decrease in these values using the following formula to investigate the extent of decrease in these values in the DM group as compared with the normal group: \(\{1 – (values \ of \ the \ DM \ group)/(values \ of \ the \ normal \ group)\} \times 100\%\).

Results

1. Fasting blood glucose levels and statistical analyses

The fasting glucose level was 83.33 ± 10.56 mg/dL.
in the normal group and 218.83 ± 35.00 mg/dL in the DM group. The fasting glucose levels were found to be significantly higher in the DM group than in the normal group (Fig. 4, Table 1).

2. Image and statistical analyses

1) Observations of light microscopic specimens

The mucosal epithelium on the lingual dorsum consisted of the stratified squamous epithelium, and keratinized, granular, stratal, and basal layer structures were observed in the epithelium of both groups. The lamina propria was observed below the epithelial layer. No infiltration of inflammatory cells was detected in the connective tissue of the lamina propria in either group (Fig. 5A–H).

2) Image and statistical analyses of light microscopic specimens

(a) Height of connective tissue papillae

The average height of the CTPe was 19.26 ± 2.34 μm in the normal group and 16.74 ± 1.99 μm in the DM group. These values were significantly lower in all the zones of the DM group than in those of the normal group (Fig. 6A, Table 2).

(b) Cross-sectional area of connective tissue papillae

The average cross-sectional area of the CTPe was 377.91 ± 53.69 μm² in the normal group and 289.45 ± 50.43 μm² in the DM group. These values were significantly lower in all the zones of the DM group than in those of the normal group (Fig. 6B, Table 2).

3) Observations of scanning electron microscopic specimens

The CLs in the CTPe were observed to incline toward the pharyngeal side in both groups. The ascending crus in the CTPe diverged superiorly from the arteriole, the CL was shaped like a hairpin loop, and the descending crus poured inferiorly into the venous network beneath the CTPe (Figs. 7A–H and 8A–H).

4) Image and statistical analyses using scanning electron microscopic specimens

(a) Number of capillary loops

There was an average of 10.92 ± 0.90 CLs in the normal group and 10.41 ± 0.98 CLs in the DM group. There was no significant difference in the number of CLs between both groups. When the number of CLs in each zone was compared between the groups, no significant decreases were detected (Fig. 9A, Table 2).

(b) Interval between tops of two capillary loops

The intervals between the tops of two adjacent CLs were 84.50 ± 7.99 and 78.86 ± 7.15 μm in the normal and DM groups, respectively. We could not detect any significant differences in the number of CLs in all the zones between both groups (Fig. 9B, Table 2).

(c) Thickness of capillary loops

The average thickness of the CLs was 9.92 ± 0.56 μm and 7.66 ± 0.53 μm in the normal and DM groups, respectively. These values were significantly lower in all the zones of the DM group than in those of the normal group (Fig. 9C, Table 2).
(4) Height of capillary loops
The average height of the CLs was 89.54 ± 2.02 μm and 73.73 ± 4.25 μm in the normal and DM groups, respectively. These values were significantly lower in all the zones of the DM group than in those of the normal group (Fig. 9D, Table 2).

(5) Crossing ratio between the ascending and descending crura of capillary loops
The average CRC of the CLs was 20.03 ± 1.29% and 13.91 ± 0.19% in the normal and DM groups, respectively. These values were significantly lower in all the zones in the DM group than in those of the normal group (Fig. 9E, Table 2).

3. Comparison of the rate of decrease in the four zones
The decrease in the height of the CTPe was the highest in the middle zone (15.80%). Those of the cross-sectional area of the CTPe were the highest in the ante-
The decrease in the thickness of the CLs was the highest in the middle zone (29.32%). The decrease in the height of the CLs was the highest in the middle zone (19.32%). The decrease in the CRC of CLs was the highest in the anterior zone (19.76%) (Table 3).

Discussion

The CTPe of filiform papillae showed simple conical shapes, and the CLs were shaped like simple hairpin loops in the CTPe from the lingual apex to the prominence on the lingual dorsum of the rats. We observed morphological differences in the CTPe and CLs in the filiform papillae between the normal and the DM groups. Further, we equally divided the lingual dorsum into four zones and investigated the prominent zones in which DM caused regressive changes in the filiform papillae.

In the following sections, we attempt to discuss the comparison of the following parameters between the normal and DM groups: 1. the connective tissue papillae, 2. the capillary loops, and 3. the decrease in the values in the four zones.

1. Connective tissue papillae

Golub et al. (1978) reported that the DM stimulated the collagenase synthesis in the gingival tissues of type 1 DM model rats. Schneir et al. (1998) reported that DM decreased collagen synthesis in the mentioned rats. Kanemura et al. (2007) concluded that DM causes regressive change in the gingival CTPe of GK rats.

Our results of the image and statistical analyses of the light microscopic specimens indicated that the both the

Table 2. Comparison in normal (N) and diabetes mellitus (DM) groups (*: p < 0.05). CTPe: connective tissue papillae, CLs: capillary loops CRC: crossing ratio between the ascending and descending crura, ①: apex zone, ②: anterior zone, ③: middle zone, ④: posterior zone.

<table>
<thead>
<tr>
<th>N groups</th>
<th>CTPe</th>
<th>Height (μm)</th>
<th>Cross-sectional area (μm²)</th>
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<tbody>
<tr>
<td></td>
<td>Average</td>
<td>①</td>
<td>②</td>
</tr>
<tr>
<td></td>
<td>19.26±2.34</td>
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<td></td>
<td>377.91±53.69</td>
<td>204.09±33.70</td>
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<tr>
<td>CLs</td>
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<td></td>
<td>Interval (μm)</td>
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<td></td>
<td>Thickness (μm)</td>
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<td></td>
<td>Height (μm)</td>
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<td></td>
<td>CRC (%)</td>
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<table>
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<td>②</td>
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<td></td>
<td>16.74±1.99*</td>
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<td>289.45±50.43*</td>
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<td></td>
<td>Interval (μm)</td>
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<td></td>
<td>Thickness (μm)</td>
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<td>Height (μm)</td>
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<td></td>
<td>CRC (%)</td>
<td>13.91±0.19*</td>
<td>8.78±0.87*</td>
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mean ± SD
Fig. 7A–H. Scanning electron micrograph of microvascular corrosion casts of lingual dorsum in normal (N) and diabetes mellitus (DM) groups. Arrow head: ascending crus, arrow: descending crus, *: venous network, ①: apex zone, ②: anterior zone, ③: middle zone, ④: posterior zone, scale bar = 50 μm.
Fig. 8A–H. Scanning electron micrograph of microvascular corrosion casts of sagittal section in normal (N) and diabetes mellitus (DM) groups. Arrow head: ascending crus, arrow: descending crus, *: capillary network, ①: apex zone, ②: anterior zone, ③: middle zone, ④: posterior zone, scale bar = 50 μm.
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height and the cross-sectional area of CTPe were significantly lower in the DM group than in the normal group (Table 3). Therefore, we concluded that DM also caused regressive change in the CTPe of the filiform papillae on the lingual dorsum in GK rats.

We considered the relationship between the blood glucose level and the fibroblasts as a possible cause of regressive changes in the CTPe in the lingual dorsum of the DM group. An in vitro study has reported that dermal fibroblast proliferation was suppressed in GK rats when the cells were cultured in media containing a high glucose content of 25.5 mM/L (459.40 mg/dL)\(^{(10)}\). In our in vivo experimental system, results indicated that the

**Table 3. Comparison of the decrease in parameters in the four zones.**

<table>
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<tr>
<th></th>
<th>Average</th>
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<th>②</th>
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<tr>
<td>CTPe Height</td>
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<td>39.28</td>
<td>31.02</td>
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<tr>
<td>Average</td>
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<td>15.08</td>
<td>19.76</td>
<td>18.51</td>
<td>14.37</td>
</tr>
</tbody>
</table>

\(^{(10)}\)
fasting blood glucose level of the DM group was 218.83 ± 35.00 mg/dL (12.15 ± 1.94 mM/L). The fasting blood glucose levels indicated the minimum values in the daily variation of the blood glucose level. Therefore, we concluded that the high concentration of the fasting blood glucose levels inhibited the proliferation of the fibroblasts in the DM group.

2. Capillary loops

By examining the microvascular corrosion casts of type 1 DM model rats, it has been reported that the microangiopathy characteristically occurred in the cheek, the tongue, and the inner marginal epithelium of the gingiva. Our results of the image and statistical analyses using the scanning electron microscopic specimens indicated that no significant differences were detected in the number of the CLs and the interval between tops of two CLs. However, the thickness and height of the CLs were significantly lower in the DM group than in the normal group (Table 3). Therefore, it has been clarified that microangiopathy occurred in the CLs of the filiform papillae in the lingual dorsum in the GK rats.

The complexity of the CLs was evaluated by the increase in the numbers, the bridging, the twisting, and the meandering shapes between the ascending and descending crura. In this study, we focused on the twisting between two crura, and calculated the CRC in each rat to quantify and analyze the complexity of the CLs. Our results of the image and the statistical analyses also indicated that the CRC were significantly lower in the CLs of the DM group than in those of the normal group (Table 3), and the CLs in the former group exhibited simple morphology.

Therefore, the CLs of the filiform papillae in the lingual dorsum of the GK rats showed regressive changes by the microangiopathy and the simplification of their morphology. As a result of the poor blood supply in the CLs, DM can be considered to cause the regressive change in the CTPe.

3. The rate of decrease in the four zones

Our results of the decrease indicated that the height of the CTPe and the thickness and height of the CLs were the highest in the middle zone (Table 3). DM caused the strongest regressive change in the CTPe and the CLs in the middle zone of the lingual dorsum in the GK rats.

On the other hand, macroscopic findings have revealed that 26.9% of DM patients showed central papillary atrophy on the lingual dorsum. Since the lingual papillae of the rats were very small, macroscopic findings did not reveal if DM caused a regressive change in the lingual papillae. However, it was suggested that similar to the case in human DM patients, DM caused the strongest regressive change in the lingual papillae of the middle zone in DM rats.

Conclusion

We equally divided the lingual dorsum from the lingual apex to the prominence into the following four zones–apex, anterior, middle, and posterior–to observe the CTPe and the CLs on the lingual dorsum in GK rats. The following results were obtained upon comparison with the normal group.

1. DM caused regressive change in the CTPe because both the height and the cross-sectional area of the CTPe were significantly lower in the DM group than in the normal group.
2. DM caused regressive change in the CLs by microangiopathy and the simplification of their shapes because the thickness, height, and CRC were significantly lower in the DM group than in the normal group.
3. DM caused the strongest regressive change in the CTPe and CLs in the middle zone of the lingual dorsum in GK rats.

Acknowledgments

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References

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