Wound healing on palatine mucosa in the type 2 diabetes mellitus model rats

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We analyzed histological changes during spontaneous healing in palatine mucosa lesions and examined whether there were any differences in healing between GK (diabetes group) and normal Wistar (control group) rats. Forty-three 8-week-old male rats (21 in each group) were used in this study. We created a cylindrical lesion 1.5 mm in diameter in the palatine mucosa of the right maxillary first molar by removing all of the soft tissue. One Wistar rat was not given a lesion and was observed about normal histological structures. Immediately and one day after surgery, the experimental cavities of both groups were filled with blood clots. The mucosal epithelium converged on day 3 after surgery in the control group and on day 7 in the diabetes group. Granulation tissue was seen on day 5 in the control group and on day 7 in the diabetes group. Collagen fibers appeared on day 7 in the controls and on day 14 in the diabetes group. We observed the formation of sequestrum on days 14 and 28 in one of six rats in the control group and five of six in the diabetes group. Our results demonstrate that wound healing was delayed in the diabetes group compared with the controls. Furthermore, sequestrum formation occurred at a higher rate in the diabetes group. (J Osaka Dent Univ 2011; 45: 37–53)

Key words: Wound healing; Palatine mucosa; Type 2 diabetes; GK rat

INTRODUCTION

When gingival grafting surgery is performed in implant and periodontal treatment, the graft is often extracted from the palatine mucosa to achieve attached gingiva.1 Since most Japanese diabetic patients have the type II disease, dentists will often encounter the need for graft surgery on these patients. Rat models are often used in studies of wound healing in diabetes. Type I diabetes, which is associated with high blood glucose levels, is typically induced by administering streptozotocin (STZ).2 Indeed, the only study on wound healing of the palatine mucosa in diabetes used STZ-administered mice,3 and reported that the healing was markedly delayed. There is no previously reported study on wound healing in the palatine mucosa using Goto-Kakizaki rats (GK rats), a model rat with spontaneous type II diabetes.4 Blood glucose levels in GK rats are lower than in STZ-administered rats.

In this study, we introduced a lesion in the palatine mucosa of GK and normal rats. The lesion had a diameter of 1.5 mm, which corresponds to the mesiodistal dimension of the maxillary first molar. We analyzed histological changes during the spontaneous healing phases and examined whether there were any differences in healing between the control and diabetes groups.

MATERIALS AND METHODS

Experimental animals

A total of 43 rats were used in this study (Shimizu Laboratory Supplies Co., Ltd., Kyoto, Japan). Twenty-two normal 8-week-old male Wistar rats were used in the control group, while 21 GK rats of the same age were used in the diabetes group. One Wistar rat was not given a lesion and was observed about normal histological structures under a light microscope. This experiment was approved by
the Osaka Dental University Animal Research Committee (Approval Numbers 07-22001, 08-02016, 09-02009) and complied with the guidelines for animal experiments.

Methods
Lesions
After measuring body weight, the rats were placed under inhalation anesthesia with isoflurane (Forane®; Abbott Japan Co., Ltd., Tokyo, Japan). In each of 21 rats of the two groups, using a biopsy trephine (Disposable Biopsy Punch®; Kai Industries Co., Ltd., Gifu, Japan), we created a cylindrical lesion 1.5 mm in diameter in the palatine mucosa near the right maxillary first molar, removing all soft tissue, including the periosteum. The lesion was intended to mimic an open wound. The site was determined as follows (Fig. 1). First, we drew a median line in the palatum. Second, a tangential line parallel to the median line was drawn at the maximum projection of the palatal surface of the first molar, and the midline between the median and the tangential lines was designated as line A. Third, another two tangential lines perpendicular to line A were drawn at the maximum projection of the mesial and the distal surfaces of the first molar. The midline between the two tangential lines was designated as line B. Finally, the intersection of lines A and B was defined as point C, which we designated as the center of the lesion. After healing periods of immediately, 1, 3, 5, 7, 14, or 28 days after surgery, three rats from each group were sacrificed using the procedures described below.

Preparation of specimens
All rats were deprived of food for 24 hours prior to the procedure. After measuring body weight, 1 mL of heparin sodium (Novo Heparin Injection 1000®; Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) was administered intraperitoneally under inhalation anesthesia with Forane®. Thirty minutes after administration, the rats were euthanized by intraperitoneal administration of excess pentobarbital sodium (Nembutal®; Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) under inhalation anesthesia. The chest was then opened, and blood was drawn from the left ventricle. The descending aorta was subsequently ligated by inserting a cannula into the ascending aorta through the left ventricle.

Perfusion was carried out using normal saline solution, and blood was removed from the right atrium. Perfusion fixation with 4% formaldehyde solution (Formaldehyde Solution®; Kishida Chemical Co., Ltd., Osaka, Japan) was carried out through the ascending aorta. The palatine region was then removed in one piece and, using the same fixation solution, was subjected to immersion fixation at 4°C for 24 h. Subsequently, decalcification was carried out for 10 days with 10% ethylenediaminetetraacetic acid solution (Ethylenediaminetetraacetic Acid Disodium Salt, 2-Hydrate®; Kishida Chemical Co.) in a microwave processor (ML-77; Azumaya Co., Tokyo, Japan). After decalcification, serial frozen frontal tissue sections of 20 µm thickness were made from the palatum using a cryostat (HM 500-OM; Carl Zeiss Japan, Tokyo, Japan). The sections started from the mesial surface layer of the right maxillary first molar and progressed in the distal direction. Hematoxylin and eosin double staining was carried out using conventional methods.

The prepared specimens were observed under a light microscope (BZ-9000; Keyence Co., Osaka, Japan).
Japan) to produce digital images. In recording healing status, we referred to the cylindrical defect as the experimental cavity. We also sub-divided the cavity structure into three parts and designated them, viewed from the surface layer, as the top-, mid-, and bottom-cavity regions. The groove seen in the maxillary bone was referred to as the groove region (Figs. 2 and 3).

Fasting blood glucose and HbA1c measurements

Fasting blood glucose
After centrifugation of blood drawn from the left ventricle, serum was collected and fasting blood glucose levels were measured using a Quick Auto Neo GLU-HK (Sino Test Co., Ltd., Tokyo, Japan).

Following the findings of Goto et al., GK rats with fasting blood glucose levels greater than 150 mg/dL were used as the diabetes group.

HbA1c
HbA1c levels in the drawn blood were measured using a Rapidia Auto HbA1c-L (Fujirebio, Inc., Tokyo, Japan). Measured values are expressed as mean ± standard deviation. Statistical analyses between groups were conducted using Student's t-test with the significance level at 1%.

RESULTS

Body weight
Body weights were 219.05 ± 50.78 g and 183.81 ± 44.77 g in the control and diabetes groups, respectively.

Fasting blood glucose and HbA1c levels
Fasting blood glucose levels were 126.05 ± 19.76 mg/dL and 187.45 ± 23.88 mg/dL in the control and diabetes groups, respectively. Fasting blood glucose levels in the controls were significantly lower than in the diabetes group (p < 0.01). HbA1c levels were 4.5% and 4.7% in the control and diabetes groups, respectively, with no significant difference between the two (p > 0.01).

Microscopy Findings
Before surgery
Mucosal epithelium and lamina propria were observed. Palatine artery, vein and nerve were observed in lamina propria. The periosteum were observed on the bone surface of the maxilla (Fig. 3).

Immediately after surgery
Blood clots were observed immediately after surgery in the experimental cavity in both groups. No palatine artery, vein, or nerve were visible in the groove region (Figs. 4A and B).

Day 1 after surgery
The experimental cavities were filled with clots rich with fibrin and red blood cells in both groups. A high level of inflammatory cell invasion, led by leu-
Fig. 4 (A) Immediately after surgery in the controls (Bar : 200 μm). The experimental cavity is filled with blood clot (+). (B) Immediately after surgery in the diabetes group (Bar : 200 μm). The experimental cavity is also filled with clot (+).

Fig. 5 (A) Day 1 after surgery in the controls (Bar : 200 μm). The experimental cavity is filled with blood clot (+). (B) Day 1 after surgery in the diabetes group (Bar : 200 μm). The experimental cavity is filled with clot (+).

kocytes, was observed in the cavities (Figs. 5A and B).

Day 3 after surgery
In the control group, crusts remained in the top-cavity region. In the mid- and bottom-cavity regions, mucosal epithelium had initiated from the periphery of the top-cavity region and moved along the cavity wall, down to the center of the bottom-cavity region, where the epithelium converged with mucosal epithelium coming from the opposite side of the cavity periphery (Fig. 6A1). In the bottom-cavity and groove regions beneath the mucosal epithelium, we observed clots that were deeply stained by eosin and consisted of tightly packed fibrin molecules. Within the clots, we found small numbers of red blood cells, leukocytes, macrophages, and even smaller numbers of lymphocytes (Fig. 6A2).

In the diabetes group, the mucosal epithelium had started from the periphery of the top-cavity region and moved along the wall, down to the mid-cavity region. However, the epithelium had not converged with mucosal epithelium from the opposite side. At the cavity center, we observed clots con-
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Fig. 6 (A1) Day 3 after surgery in the controls (Bar: 200 μm). Mucosal epithelium converged with mucosal epithelium coming from the opposite side at the center of the bottom-cavity region. (A2) Higher magnification of the bottom-cavity region in A1. Leukocytes (▲), macrophages (▲) and lymphocytes (→) are observed in the clot. (B1) Day 3 after surgery in the diabetes group (Bar: 200 μm). Mucosal epithelium had not converged with mucosal epithelium coming from the opposite side. (B2) Higher magnification of bottom-cavity region in B1. Leukocytes (▲), macrophages (▲) and lymphocytes (→) are observed in the clot.

sisting of red blood cells and tightly packed fibrin molecules deeply stained by eosin; they were seen from the top-cavity region to the groove region (Fig. 6B1). Within the clots, we found small numbers of leukocytes and macrophages, and a smaller number of lymphocytes; these were particularly numerous at the periphery of the bottom-cavity region (Fig. 6B2).

Day 5 after surgery
In the control group, no crust was observed in the top-cavity region, the center of which was depressed. Converged, continual mucosal epithelium had risen to the mid-cavity region. A thin stratum corneum was seen at the surface layer of the mucosal epithelium (Fig. 7A1). In the mid-cavity, bottom-cavity, and groove regions underneath the mucosal epithelium, we observed granulation tissue with numerous fibroblasts and a small number of new blood vessels. We found small numbers of leukocytes, macrophages, and an even smaller number of lymphocytes inside the granulation tissue. Residual clots were visible in parts of the groove region (Fig. 7A2).

In the diabetes group, the mucosal epithelium had progressed from the periphery of the top-cavity
Fig. 7 (A1) Day 5 after surgery in the controls. Epithelium (E) (Bar: 200 μm). (A2) Higher magnification of the bottom-cavity region in A1. Fibroblasts (→), leukocytes (●), macrophages (◇) and lymphocytes (▲) are observed in the clot. (B1) Day 5 after surgery in the diabetes group (Bar: 200 μm). Mucosal epithelium had not converged with mucosal epithelium coming from the opposite side. (B2) Higher magnification of the mid-cavity region in B1. Inflammatory cell invasion that included leukocytes (●), macrophages (◇) is observed. (B3) Higher magnification of the groove region in B1. Inflammatory cell invasion that included leukocytes (●), macrophages (◇) and lymphocytes (▲) is observed.

region down to the bottom-cavity along the wall. However, we saw no convergence with mucosal epithelium from the opposite side in the top-cavity region (Fig. 7B1). Crusts were visible at the center of the top-cavity region. At the center of the mid-cavity region, we found a high level of inflammatory cell invasion, including necrotized cells, leukocytes, and macrophages (Fig. 7B2). Blood clots were
Fig. 8 (A1) Day 7 after surgery in the controls. Epithelium (E) (Bar: 200 μm). (A2) Higher magnification of the mid-cavity region of A1. Fibroblasts (−), leukocytes (●) and lymphocytes (++) are observed. (A3) Higher magnification of the groove region in A1. Newly formed small-caliber blood vessels (V) are observed. (B1) Day 7 after surgery in the diabetes group. Epithelium (E) (Bar: 200 μm). Mucosal epithelium converged with mucosal epithelium coming from the opposite side. (B2) Higher magnification of the mid-cavity region in B1. Fibroblasts (−), leukocytes (●), macrophages (●) and lymphocytes (++) are observed. (B3) Higher magnification of the groove region in B1. Newly formed small-caliber blood vessels (V), leukocytes (●), macrophages (●) and lymphocytes (++) are observed.
Fig. 9  (A1) Day 14 after surgery in the controls showing epithelium (E) and a neurilemma-like structure (N) (Bar: 200 μm).  (A2) Higher magnification of the mid-cavity region in A1. Fibroblasts (→) and an epithelial processes (E) are observed. (A3) Higher magnification of the groove region in A1. Newly formed small-caliber vessels (V), a neurilemma-like structure (N) and periostium like structures (P) are observed. (B1) Day 14 after surgery in the diabetes group showing epithelium (E) and a sequestrum (S) (Bar: 200 μm).  (B2) Higher magnification of the mid-cavity region in B1 showing fibroblasts (→).  (B3) Higher magnification of the bottom-cavity region in B1 showing fibroblasts (→) and small-caliber blood vessels (V).  (B4) Higher magnification of the black framed area in B1 showing a sequestrum (S), leukocytes (←), macrophages (→) and lymphocytes (→).
Fig. 10A (A1) Day 28 after surgery in the controls. Epithelium (E) (Bar: 200μm). A neurilemma-like structure (N) is observed. (A2) Higher magnification of the mid-cavity region in A1 showing fibroblasts (○) and small-caliber blood vessels (V). (A3) Higher magnification of the groove region in A1 showing fibroblasts (○) and small-caliber blood vessels (V). (A4) Higher magnification of the groove region in A1 showing a neurilemma-like structure (N). (AS1) Day 28 after surgery in the controls with a sequestrum (S). Epithelium (E) (Bar: 200μm). (AS2) Higher magnification of the top-cavity region in AS1 showing fibroblasts (○) and small-caliber blood vessels (V). (AS3) Higher magnification of the groove region in AS1 showing a neurilemma-like structure (N). (AS4) Higher magnification of the black framed area in AS1 showing a sequestrum (S), leukocytes (▲), macrophages (●) and lymphocytes (←).
Fig. 10B  (B1) Day 28 after surgery in the diabetes group showing a epithelium (E), a neurilemma-like structure (N) and cyst-like structures (C) (Bar: 200 μm). (B2) Higher magnification of the top-cavity region in B1 showing fibroblasts (○). (B3) Higher magnification of the mid region in B1 showing cyst-like structures (C). (B4) Higher magnification of the groove region in B1 showing blood vessels (V), a neurilemma-like structure (N) and periosteum-like structures (P). (BS1) Day 28 after surgery in the diabetes group with a sequestrum (S). A neurilemma-like structure (N) is observed. Epithelium (E) (Bar: 200 μm). (BS2) Higher magnification of the top-cavity region in BS1 showing fibroblasts (○) and small-caliber blood vessels (V). (BS3) Higher magnification of the groove region in BS1 showing small-caliber blood vessels (V), a neurilemma-like structure (N) and periosteum like structures (P). (BS4) Higher magnification of the black framed area in BS1 showing a sequestrum (S), leukocytes (●), macrophages (○) and lymphocytes (●).
seen in the bottom-cavity region. At the periphery of the bottom-cavity region and in the groove region, we found a high level of inflammatory cell invasion, consisting of many leukocytes, macrophages, and a small number of lymphocytes (Fig. 7 B3). Overall, few blood vessels were seen in the cavity.

Day 7 after surgery
In the controls, a continuous mucosal epithelium had risen to the top-cavity region. A thin stratum corneum and epithelial processes appeared at the surface layer of the mucosal epithelium. The morphology of the epithelial processes was conical around the cavity periphery and was trapezoidal with a conical process at the cavity center. The number of epithelial processes was small, their lengths short, and they were confined within the top-cavity region. The epithelial processes found at the cavity center were elongated up to the boundary between the top-cavity and mid-cavity regions (Fig. 8A1). Numerous fibroblasts and a small number of collagen fibers were visible in the entire cavity underneath the mucosal epithelium. The thickness of granulation tissue had increased, compared with that seen on day 5. Only a very small number of leukocytes and lymphocytes were found in the lamina propria mucosa (Fig. 8A2). We also found a small number of newly formed small-caliber blood vessels (Fig. 8A3).

In the diabetes group, no crust was seen in the top-cavity region. In the mid-cavity region, convergence of mucosal epithelium had occurred at the cavity center; the convergence had not reached the top-cavity region. A thin stratum corneum and epithelial processes appeared at the surface layer of the mucosal epithelium. The morphology of the epithelial processes was conical at the cavity periphery; they appeared as one large trapezoid at the cavity center. The number of epithelial processes was small; their lengths were short, and they were confined within the mid-cavity region (Fig. 8B1). In the mid- and bottom-cavity regions, as well as the groove region underneath the mucosal epithelium, we saw granulation tissue containing many fibroblasts. In the mid- and bottom-cavity regions underneath the mucosal epithelium, we observed a mild level of inflammatory cell invasion, consisting of small numbers of leukocytes, macrophages, and a very small number of lymphocytes (Fig. 8B2). In the groove region, many newly formed small-caliber blood vessels had appeared. At the bone surface of the palatine process of the maxilla, which constituted the floor of the experimental cavity, we found small numbers of leukocytes and macrophages, and a smaller number of lymphocytes. On the bone surface, signs of bone resorption were seen, extending to the root side, the nasal passage, and the median line (Fig. 8B3). On the apical side of the alveolar bone located at the palatine side of the first molar root, we saw signs of dish-shaped bone resorption, extending from the root side to the median line. Within the bone resorption, we also observed invasion of inflammatory cells, such as primarily leukocytes.

Day 14 after surgery
In the control group, the mucosal epithelium had bulged prominently above the top-cavity region across the width of the cavity. The thickness of the mucosal epithelium had increased compared with day 7. There were numerous bumpy convexoconcave structures and, compared with day 7, the stratum corneum at the surface layer of mucosal epithelium had thickened. Epithelial processes were visible in the mucosal epithelium; their morphology was rod-shaped and they were aligned and evenly spaced. The number of epithelial processes had increased relative to that on day 7; they were elongated towards the lamina propria and had reached the boundary of the mid- and bottom-cavity regions (Fig. 9A1). In the lamina propria of the mid-cavity region, we saw protrusion of connective tissue papilla, corresponding to the asperity of the mucosal epithelium. Many fibroblasts and collagen fibers were found in the lamina propria of the mid- and bottom-cavity regions, and in the groove region (Fig. 9A2). Numerous small-caliber blood vessels were visible in the mid- and bottom-cavity regions, as well as in the groove region. In the groove
region, we found a neurilemma-like structure (Figs. 9A1 and A3). The lamina propria was almost free of inflammatory cells. Periosteum-like structures were observed on the bone surfaces in the bottom-cavity and groove regions (Fig. 9A3). These structures consisted of one layer of cube-shaped cells stained with hematoxylin, small-caliber blood vessels on the bone surface, and a layer of fibroconnective tissue at the side of the mucosal epithelium. The periosteum-like structures were continuous with the structures on the surface of bone immediately adjacent to the lesion site. A layer lightly stained with eosin was visible in the bone directly underneath the periosteum-like structures. We found no bone resorption on the bone surface constituting the floor of the experimental cavity. No sequestrum was observed in any of the three animals.

In the diabetes group, the mucosal epithelium had reached the top-cavity region, while its thickness was approximately the same as on day 7. We saw a thin stratum corneum at the surface layer of the mucosal epithelium, and it looked roughly similar to that seen on day 7. Epithelial processes were visible in the mucosal epithelium; they showed a conical morphology and the alignment was unevenly spaced. As on day 7, the number of epithelial processes was small and they had reached the boundary of the top- and mid-cavity regions (Fig. 9B1). In the mid-cavity region under the mucosal epithelium, we observed granulation tissue containing many fibroblasts and collagen fibers (Fig. 9B2). In the bottom-cavity and groove regions, we found granulation tissue containing many fibroblasts and a small number of collagen fibers, and also found many small-caliber blood vessels (Fig. 9B3). A neurilemma-like structure was visible in the groove region. In the palatal process of the maxilla, which constitutes the floor of the experimental cavity, we saw signs of marked bone resorption on the bone surface, at the root side, adjacent to the nasal passage, and on the side of the median line. Bone resorption had spread from the alveolar bone on the palatal side of the root towards the median line, penetrating through the palatine process of the maxilla. Because of bone resorption, a dissociated sequestrum lacking bone cells was found in the bone lacuna. Surrounding the sequestrum, we observed a high level of inflammatory cell invasion consisting primarily of leukocytes, macrophages, and a few lymphocytes, around which granulation tissue was visible (Figs. 9B1 and B4). A sequestrum was detected in all three of the experimental animals.

Day 28 after surgery
In the control group, one rat had sequestrum, while the other two did not.

Control group without sequestrum
In the two rats without sequestrum, the mucosal epithelium had gone past the top-cavity region at the cavity center and bulged towards the side of the mucosal epithelium. The thickness of the mucosal epithelium and its stratum corneum had become thinner than on day 14. Epithelial processes were found in the mucosal epithelium; their morphology was rod-shaped and aligned with even spacing. The number of epithelial processes was greater than on day 14, while their length had become shorter. They reached the top- and mid-cavity regions at the center and periphery of the cavity, respectively (Fig. 10A1). The lamina propria was thick at the cavity center, reaching the upper end of the top-cavity region, and had bulged to the side of the mucosal epithelium. At the cavity periphery, however, it was thinner than at the cavity center and had not bulged. In the bulged lamina propria in the top- and mid-cavity regions, we observed a small number of fibroblasts, many collagen fibers, and many small-caliber blood vessels (Fig. 10A2). In the bottom-cavity and groove regions, we observed many fibroblasts and collagen fibers. We also found a small number of small-caliber blood vessels in the bottom-cavity region. In the groove region, we saw many large and small blood vessels (Fig. 10A3) as well as a neurilemma-like structure (Fig. 10A4). In the lamina propria, described above for each region, hardly any inflammatory cells were visible. On the bone surface of the palatine process
of the maxilla, which constitutes the floor of the experimental cavity, we observed periosteum-like structures. They consisted of one layer of cube-shaped cells stained by hematoxylin, small-caliber blood vessels, and a layer of fibroconnective tissue at the side of the mucosal epithelium. These periosteum-like structures had converged into the structures on the bone surface adjacent to the lesion site. A layer lightly stained by eosin was seen in the bone directly underneath the periosteum-like structures.

Control group with sequestrum
In the one rat with a sequestrum in the control group, the mucosal epithelium had bulged over the top-cavity region at the cavity center. The mucosal epithelium and its stratum corneum had become thinner than on day 14. Epithelial processes were visible in the mucosal epithelium; their morphology was rod-shaped, aligned, and evenly spaced. The number of epithelial processes was the same as on day 14, but their lengths had become shorter, and they had reached the top-cavity region of the cavity center and the mid-cavity region of the cavity periphery. The lamina propria was thick at the cavity center, reaching the upper edge of the top-cavity region, and bulged onto the side of the mucosal epithelium. At the cavity periphery, however, it was thinner than at the cavity center, and no bulging had occurred (Fig. 10A1). Many fibroblasts, collagen fibers, and small-caliber blood vessels were observed in the bulged lamina propria in the top-, mid-, and bottom-cavity regions, and in the groove region (Fig. 10A2). A neurilemma-like structure was observed in the groove region (Fig. 10A3). While few inflammatory cells were found in the lamina propria in the top- and mid-cavity regions, we found macrophages in the bottom-cavity and groove regions. On the bone surface in the groove region, we saw periosteum-like structures and one layer of cube-shaped cells stained by hematoxylin. We also saw a layer lightly stained by eosin in the bone directly underneath the periosteum-like structures. In the palatine process of the maxilla, which constitutes the cavity floor, a large cavity caused by bone resorption was seen with its opening on the mucous membrane side. At the center of this resorption image was a dissociated sequestrum; surrounding the sequestrum, we observed a high level of inflammatory cell invasion consisting primarily of leukocytes, as well as macrophages, and a small number of lymphocytes. In the area surrounding the inflammatory cells, we found granulation tissue containing fibroblasts and collagen fibers (Fig. 10AS4). We saw no resorption in the alveolar process of the maxillary bone.

Among the three rats in the diabetes group, two each had a sequestrum while the other did not.

Diabetes group without sequestrum
In the rat with no sequestrum, the mucosal epithelium had grown over the top-cavity region at the cavity center and had bulged out to the side of the mucosal epithelium. The mucosal epithelium and its stratum corneum were as thin as on day 14. Epithelial processes were seen in the mucosal epithelium; their morphology was rod-shaped, their sizes were variable, and their spacing were uneven. The number of epithelial processes remained the same as on day 14, although their lengths varied (Fig. 10B1). The lamina propria had grown over the top-cavity region at the cavity center and prominently bulged out to the side of the mucosal epithelium. We observed many fibroblasts and collagen fibers in the bulged lamina propria in the top-, mid-, and bottom-cavity regions (Fig. 10B2). Round cyst-like structures were observed in the lamina propria of the mid- to bottom-cavity regions. The walls of these cyst-like structures exhibited a dual-layer structure. The outer layer consisted of three or four layers of cells that looked like stratified squamous epithelium and were stained with hematoxylin, but it did not exhibit a structure typical of normal stratified squamous epithelium. The inner layer of the wall consisted of cuticle-like structures stained by eosin. Inside the cyst-like structures was a cluster of cuticle-like structures aggregating in a concentric manner (Fig. 10B3). There were numerous blood vessels in the protruding region, going from the top-cavity region to the mucosal epithelium side, as
well as in the bottom-cavity and groove regions. Within the lamina propria, described above for each region, small numbers of neutrophils and histiocytes were visible. A neurilemma-like structure was also observed in the groove region. Hematoxylin staining revealed periosteum-like structures and one layer of cube-shaped cells on the bone surface in the groove region (Fig. 10B4). A layer lightly stained by eosin was also visible in the bones directly beneath the periosteum-like structures. In the palatine process of the maxilla, which constitutes the floor of the experimental cavity, a large cavity caused by bone resorption was seen, with two openings, one on the mucous membrane side and the other on the root side. At the center of this resorption was a dissociated sequestrum. In the area surrounding the sequestrum, we observed a high level of inflammatory cell invasion consisting primarily of leukocytes together with a small number of lymphocytes and macrophages. We also observed a small quantity of granulation tissue in the area surrounding the inflammatory cells (Fig. 10BS4).

**DISCUSSION**

**Experimental animals**

In many of the animal experiments on diabetes mellitus conducted to date, rats were used in which streptozotocin (STZ) had been administered to induce type I diabetes. However, some reports have indicated that STZ administration can affect liver and kidney function. Therefore, we thought it quite possible that STZ might also affect wound healing. To investigate the effect of high blood glucose alone on wound healing, i.e., in the absence of possible effects on the liver and kidney caused by the drug, we used rats with spontaneous type II diabetes as a model system.

According to Japan SLC (2007), rats administered STZ show an extremely high blood glucose level, 423~600 mg/dL, caused by the selective destruction of pancreatic islet β-cells. As a result, these rats are thought to exhibit features of severe diabetes. However, it has been reported that blood glucose levels in GK rats, a rat model with spontaneous type II diabetes, exceed 150 mg/dL. In this study, the blood glucose level of GK rats was 187.45 ± 23.88 mg/dL, a level high enough to ensure diabetes mellitus. Some studies have reported a delay in wound healing using STZ-administered rats. However, no studies exist on wound healing in the palatine mucosa of GK rats, the blood glucose levels of which are approximately one-third of that
in STZ-administered rats. In the current study, we used GK rats to investigate changes in wound-healing and to examine whether the healing period might be delayed in GK rats compared to normal rats.

Lesion size
Wound healing is markedly delayed in STZ-administered mice. The lesion made by Sakoh in STZ-administered mice was large, and involved exfoliating and removing the entire mucosa of the hard palate between the left and right molars. Such a procedure is not used in conventional clinical dentistry. In fact, the mesiodistal diameter of the premolars is used as a landmark for the size of the mucosal flap in clinical practice. In the present study, we introduced a lesion using the mesiodistal width of the maxillary first molar as a landmark, and set the lesion size accordingly to 1.5 mm in diameter. The lesion that we created was thus much smaller than that made by Sakoh. Nevertheless, we found that wound healing of the mucosal epithelium and the lamina propria was delayed in the diabetes group compared to the controls. It is thus likely that wound healing would be delayed regardless of lesion size in diabetics.

Comparison of wound healing
Below, we compare wound healing in the mucosal epithelium and lamina propria between the control and diabetic groups, and also discuss other structures and sequestrum.

Wound healing of the mucosal epithelium
The mucosal epithelium converged on day 3 after surgery in the control group and on day 7 in the diabetes group. Thus, wound healing appeared to be delayed in the diabetes group. Epithelial cells need to migrate in order for mucosal epithelium to cover a lesion. In an in vitro system, it was reported that migration of oral epithelial-derived cells is markedly inhibited under high-glucose conditions compared to migration under lower glucose conditions. Expression of integrin, which is necessary for connection with the extracellular matrix, was also stimulated and cell migration suppressed under high glucose conditions. Thus, it is likely that cell migration necessary to cover the lesion was suppressed under high blood glucose conditions, potentially explaining why healing of the mucosal epithelium was delayed in diabetic rats.

Wound healing of lamina propria
Although granulation tissue was seen on day 5 in the controls, it was seen on day 7 in the diabetes group. It was previously reported that the ability of fibroblasts to produce glycosaminoglycan was reduced at high glucose levels and that sufficient granulation tissue formation did not occur. This is likely why the emergence of granulation tissue was delayed in the diabetes group compared with the controls.

Although collagen fibers appeared on day 7 in the controls, they appeared on day 14 in the diabetes group. The proliferation capacity of fibroblasts has been reported to be reduced at high glucose levels. Furthermore, collagen production capacity decreases and the activity of collagen-digesting enzymes increases in the gingival tissues of STZ-administered rats. This may explain why the appearance of collagen fibers was delayed in the diabetes group compared with the controls. Thus, both the proliferative capacity of fibroblasts and production capacity of collagen fibers decrease under high blood glucose conditions. This may explain why wound healing in the lamina propria was delayed in diabetic rats.

Cyst-like structures
We observed cyst-like structures on day 28 in one rat in the diabetes group, i.e., the animal that did not develop sequestrum. We think that these structures were not cysts because they lacked a fibro-connective tissue wrapping and did not have a clear boundary with surrounding tissues. The outer layer of the cyst-like structures was similar to stratified squamous epithelium, and the inner layer consisted of cuticle-like structures. One possibility is that the stratified squamous epithelium-like cells in the outer layer grew and became the cuticle-like...
structures in the inner layer. The cuticle-like structures in the inner layer appeared to have no void spaces. The images of tissues showing apparent spaces could be the result of artifacts introduced during specimen preparation. Possible causes of the artifacts include shrinkage of the cuticle-like structures caused by dehydration treatment during specimen preparation, or loss of tissue when cutting thin sections. How did the cyst-like structures form? One possibility is that when the epithelium was progressing from the surroundings to the wall of the experimental cavity, part of the progressing epithelium strayed into the subepithelial area and became isolated.

Neurilemma-like structure
We observed a neurilemma-like structure in the groove region on days 14 and 28 in both groups. Before creating the lesion, there were a palatine artery, vein, and nerve in the groove region. If they had regenerated, similar structures would likely be observed at the same sites where they existed before the lesion was created. Because the neurilemma-like structure observed did not have a hollow center, there were no blood vessels. It is more likely that it was a regenerated neurilemma of the palatine nerve; we thus describe it as a neurilemma-like structure. However, we did not carry out the specific staining needed to identify nerve fibers and nerve sheaths. Further investigation is needed to address this issue.

Periosteum-like structures
The periosteum consists of fiber layers and, on the bone side, bone-forming layers rich in blood vessels. Basophilic osteoblasts forming two or three cellular layers were observed on the bone surface of the active periosteum. The cube-shaped cells observed on the bone surface on days 14 and 28 in the control group were deeply stained with hematoxylin; they were likely osteoblast cells. We also observed a layer of fibroconnective tissue, which is likely a fiber layer on the mucosal epithelium side of these cells. Because these structures were similar to those on the bone surface adjacent to the lesion site, we speculate that the periosteum had regenerated. The layer that was lightly stained by eosin and was located on the bone directly under the periosteum-like structures probably corresponded to a bone-like tissue layer. However, we did not carry out the specific staining necessary to determine whether the cube-shaped cells were osteoblasts.

Sequestrum
We observed the formation of sequestrum on days 14 and 28 in one of the six rats in the control group (17%) and five of six in the diabetes group (83%). That is, the diabetes group formed a sequestrum at a considerably higher rate than the control group.

Formation of sequestrum in the diabetes group
In the diabetes group, invading inflammatory cells, consisting primarily of leukocytes, were continuously visible from days 1 to 28. This observation indicates that suppurative inflammation persisted for a longer period in diabetic rats than in the controls. One reason for persistent suppurative inflammation could be that, in addition to increased susceptibility to infection at high blood glucose levels, drugs (e.g., antibiotics) were not administered to either group in order to allow observation of the spontaneous healing status of the lesion. During this period of continuous inflammation, bone resorption began on day 7 and a dissociated sequestrum formed on days 14 and 28. These observations support the possibility that continuous suppurative inflammation in diabetes promoted bone resorption, resulting in the formation of dissociated sequestrum.

Formation of sequestrum in the control group
Among the control group rats for which sequestrum formation was not seen, few inflammatory cells were observed from days 7 to 28, indicating that inflammation was subsiding during this period. However, in the one rat in which we did see sequestrum on day 28, we observed a high level of inflammatory cell invasion consisting primarily of leukocytes in the area surrounding the sequestrum. As described above, no drugs were administered.
In this one example, it seems likely that, just as in the rats of the diabetes group, continuous suppurrative inflammation over a long period may have caused sequestrum formation.

Taken together, these results demonstrate that wound healing in the palatine mucosa of GK rats, where the blood glucose level was approximately 180 mg/dL, was slower than in normal rats, and that sequestrum formation occurred at a higher rate. These findings support the view that, in clinical dentistry, caution must be exercised when performing surgery that could involve creating lesions in the palatine mucosa of diabetic patients.

CONCLUSIONS

An experimental lesion was introduced in the palatine mucosa of normal and GK rats. We observed histological changes in the spontaneous healing process and compared the observations with those of normal rats. Our results demonstrated that wound healing was delayed in GK rats compared with normal animals. Furthermore, sequestrum formation occurred at a higher frequency in GK rats.

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REFERENCES