INTRODUCTION

Diabetic patients have been known to be associated with a higher incidence of periodontal disease, and diabetes mellitus has been reported to be an important risk factor for periodontal disease1. At present, periodontal disease is proposed to represent the sixth most common diabetic complication, following retinopathy, nephropathy, neuropathy, microangiopathy, and macroangiopathy2. Among those diabetic complications, retinopathy, nephropathy, and neuropathy are attributed to microangiopathy, while several disease conditions, including myocardial or cerebral infarction, are caused by macroangiopathy. Thus, all diabetic complications are attributed to vascular impairment. Therefore, the high incidence of periodontal disease in diabetic patients may be partly explained by the possible presence of microcirculatory impairment in the periodontium3.

A vascular endothelial growth factor (VEGF) is a glycoprotein that promotes the growth and differentiation of vascular endothelial cells, enhances vascular permeability, and dilates blood vessels. It is one of the factors promoting an-

Relationship between VEGF and AGEs on Periodontal Wound Healing in Model Rats with Type 2 Diabetes Mellitus

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SYNOPSIS

VEGF is known to play a significant role in microangiopathy through AGEs. The objective of this study was to observe the expression and distribution of VEGF and AGEs immunohistochemically in the early healing stage of periodontal defects in model rats with type 2 diabetes (GK), and then clarify the relationship between these expressions and microangiopathy in the healing process.

We surgically prepared periodontal defects in the maxillary molars of male 30-week-old GK and SD rats. On Days 3, 5, and 7 after surgery, the rats were euthanized. The periodontal samples were stained immunohistochemically with anti-VEGF and anti-AGE monoclonal antibodies.

AGEs and VEGF were more significantly observed in the GK rats than in the SD rats around blood vessels. These results suggest that, in the GK rats undergoing periodontal surgery, AGEs localized around capillary blood vessels contribute to an intensive expression of VEGF compared to the SD rats, and then VEGF causes microangiopathy during wound healing.

Key words: VEGF, AGEs, Periodontal wound healing, Type 2 diabetes mellitus
giogenesis, for example, in the course of wound healing. VEGF is also known to be involved in normal vascularization, as well as play an important role in pathological vascularization or the conditions associated with vascular hyperpermeability, such as malignancies and diabetic retinopathy4-7.

Advanced glycation end products (AGEs) are a collective term that refers to high-molecular-weight molecules formed as a result of the nonenzymatic glycosylation and modification of proteins8, 9. The production of AGEs can occur in the blood, extracellular matrix, and throughout the inside of cells. If chronic hyperglycemia persists, as seen in diabetic patients with poorly-controlled blood glucose, AGEs can be formed and accumulate in various tissues of the body. For this reason, AGEs have drawn attention as providing a mechanism for the onset of diabetic complications. Particularly, AGEs in the receptors present on vascular endothelial cells (RAGE) are known to increase the production of oxidative stress and then induce various vascular disorders as well as the aging of blood vessels10.

However, the contribution of VEGF and AGEs to vascularization during wound healing in diabetic patients who have undergone periodontal surgery remains to be elucidated. Therefore, we prepared periodontal defects artificially in model rats with type 2 diabetes, and immunohistologically observed VEGF expression and AGE distribution during wound healing to clarify the relationship between VEGF and AGEs on vascularization during wound healing in diabetic patients.

MATERIALS AND METHODS
Experimental materials
The present study was approved by the Committee for Animal Experiments of Osaka Dental University (approval number: 11-04009), and conducted in accordance with the guidelines for animal experimentation.

The experiment employed 18 each of male GK (animal model with type 2 diabetes: body weight: 180 - 210 g; for experimental group) and Sprague Dawley (SD: 210 - 230 g; for control group) rats aged 8 weeks that were acquired from the SHIMIZU Laboratory Supplies Co., Ltd., and then raised for 24 weeks (body weight: 385 - 450 g and 515 - 675 g, respectively). The experimental group consisted of only rats that showed a fasting blood glucose level of 200 mg/dL or above in prior measurements of the level in blood collected through the caudal vein using a Nipro Free Style Flash™ (NIPRO, Osaka, Japan).

Experimental method
Preoperatively, the body weight and fasting glucose level were again measured in both experimental and control groups.

Rats were anesthetized by the inhalation of isoflurane (Forane®, Abbott, NorthChicago, IL, U.S.A.), injected intraperitoneally with 0.3 mg/kg of pentobarbital sodium (Somuno pentil injection®, Kyoritsu Seiyaku Corporation, Tokyo, Japan), and fixed in the supine position with their mouths open.

The surgery was performed according the following procedures to observe the periodontium including the central roots on the palatal side of bilateral maxillary first molars:

An internal bevel incision was made using a slit-knife (Alcon, Hünenberg, Switzerland) in the gingival crevice from the center of the surface adjacent to maxillary first molar mesial palatal side to the second molar mesial palatal angle. In the incised area, a full-thickness flap consisting of the mucosal epithelium, lamina propria, and periosteum was prepared using a dental excavator (Hu-Friedy, Chicago, IL, U.S.A.). From the maxillary first molar mesial palatal
angle to the second molar mesial palatal angle, the alveolar bone, periodontal membrane, and surface of the tooth root (cementum and dentin) were curetted away using a Mini Five Gracey Curette (#7/8, Hu-Friedy, Chicago, IL, U.S.A.) to create an artificial periodontal defect.

The surgical fields were washed fully with physiological saline. Then, the full-thickness gingival flap was returned to the initial position and the mesial first molar was single-sutured using a 6-0 absorbable thread (Biosorb C®, Alcon, Hünenberg, Switzerland) (Fig. 1). The experimental periods were 3, 5, and 7 days after surgery. Six rats each were employed for the experimental and control groups in each period.

Preparation of the samples for histopathology and immunohistochemistry

The rats of both control and experimental groups were euthanized with an overdose of Somuno pentil injection in each period. Immediately after euthanasia, the chest was incised and a catheter was inserted into the ascending aorta, through which 10% neutral formaldehyde solution (Nacalai Tesque, Kyoto, Japan) was perfused for fixation. A mass of the surrounding tissues including the experimented tooth root was collected, immersed in fresh fixation solution at 4°C for three days, and then decalcified in rapid decalcifying solution (K-CX, Falma, Tokyo, Japan) at 4°C for 24 hours.

The decalcified sample was divided in the buccal to palatal position at the observation site, i.e., the mesial side of the first molar palatal central root after removing excess tissues. The divided sample was washed using 0.1 M phosphate buffer solution (PBS; pH 7.2) at 4°C and then paraffin-embedded according the conventional method. Subsequently, serial sections with a thickness of 5 μm were prepared over a range allowing observation of the first molar palatal central root.

Immunohistochemical staining was performed with anti-VEGF monoclonal antibody (sc-7269, Santa Cruz Biotechnology, California, U.S.A.) in the sections of both experimental and control groups. For this staining, the sections were deparaffinized and then reacted with 0.4% solution of pepsin in 0.01 N HCl at 37°C for 30 minutes to activate antigens.

After inactivating endogenous peroxidase in 0.3% H2O2, the sections were reacted with anti-VEGF monoclonal antibody diluted to 400 fold with PBS at 37°C for 60 minutes, and then colored with 3,3-diaminobenzidine- tetrahydrochloride (DAB, DakoCytomation, Glostrup, Denmark) using the EnVision™++ system (DakoCytomation, Glostrup, Denmark). Subsequently, the sections were subjected to nuclear staining with hematoxylin, dehydrated, enclosed, and observed using an All-in-One Fluorescence Microscope (BZ9000, KEYENCE, Tokyo, Japan). The sections for both experimental and control groups were subjected to immunohistochemical staining with anti-AGEs monoclonal antibody (KH001-02, TransGenic Inc., Kumamoto, Japan).

For this staining, the sections were deparaffinized and then reacted with 0.4% solution of pepsin in 0.01 N HCl at 37°C for 30 minutes to activate antigens. After inactivating endogenous peroxidase in 0.3% H2O2, the sections were reacted with anti-AGEs monoclonal antibody diluted 500 fold with PBS at 37°C for 60 minutes, and then colored with DAB using the EnVision™++ system. Then, the sections were subjected to nuclear staining with hematoxylin, dehydrated, enclosed, and observed using an All-in-One Fluorescence Microscope.
**Table 1**  Body weight and blood glucose level of the rats

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<thead>
<tr>
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<th>Body weight (g)</th>
<th>Blood glucose level (mg/dl)</th>
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<tbody>
<tr>
<td>Control group</td>
<td>586.67±37.81</td>
<td>126.75±16.88</td>
</tr>
<tr>
<td>Experimental group</td>
<td>424.18±16.96</td>
<td>274.00±37.94</td>
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Mean±SD, p<0.01

**Fig. 1**  Schema of a rat’s maxilla
(Red line) : Incision line
(Yellow line) : Suture site

**Fig. 2**  Immunohistochemical staining with anti-VEGF antibody on day 3 post-surgery
A. Control group  ×100
B. Experimental group  ×100
C. Experimental group  ×400  (▵) : Expression of VEGF
En=Endothelial cells, Lu=Vascular lumen, Bc=Blood clots

**Fig. 3**  Immunohistochemical staining with anti-VEGF antibody on day 5 post-surgery
A. Control group  ×100
B. Experimental group  ×100
C. Experimental group  ×400  (▵) : Expression of VEGF
**Fig. 4** Immunohistochemical staining with anti-VEGF antibody on day 7 post-surgery
A. Control group ×100
B. Experimental group ×100
C. Experimental group ×400 (△): Expression of VEGF
GT=Granulation tissue

**Fig. 5** Immunohistochemical staining with anti-AGEs antibody on day 3 post-surgery
A. Control group ×100
B. Experimental group ×100
C. Experimental group ×400 (△): Localization of AGEs

**Fig. 6** Immunohistochemical staining with anti-AGEs antibody on day 5 post-surgery
A. Control group ×100
B. Experimental group ×100
C. Experimental group ×400 (△): Localization of AGEs

**Fig. 7** Immunohistochemical staining with anti-AGEs antibody on day 7 post-surgery
A. Control group ×100
B. Experimental group ×100
C. Experimental group ×400 (△): Localization of AGEs
RESULTS

**Body weight and blood glucose level**
The mean body weight was lower but the mean fasting blood glucose was higher in the experimental than control group (Table 1).

**Findings on immunohistochemical staining with anti-VEGF antibody**

**Day 3 post-surgery**
Blood clots consisting predominantly of fibrins, including neutrophils, in periodontal defects were observed in both control and experimental groups. In gingival connective tissues around these blood clots, capillary vascularization and vasodilation were noted. More capillary blood vessels were observed in the experimental than the control group, which had an irregular lumen (Fig. 2A, B).

VEGF expression in connective tissues around blood clots, although identified in both groups, was more intensive in the experimental group. Particularly intense expression of VEGF was observed near capillary endothelial cells in the experimental group (Fig. 2C).

**Day 5 post-surgery**
Blood clots in periodontal defects persisted in the experimental group, whereas nearly all disappeared in the control group. In both groups, granulation tissues involving the infiltration of numerous neutrophils were noted in periodontal defects. In the experimental group, more new capillary blood vessels were observed around granulation tissues and the surrounding connective tissues than in the control group; these vessels showed luminal dilation.

Minimal expression of VEGF was noted in granulation tissues and the surrounding connective tissues in the control group. In contrast, VEGF expression in blood clots and the surrounding tissues was found in the experimental group (Fig. 3A, B). An intensive expression of VEGF was observed particularly near capillary endothelial cells in connective tissues around blood clots in the experimental group, but not in the control group (Fig. 3C).

**Day 7 post-surgery**
In both groups, blood clots nearly disappeared, and periodontal defects were nearly filled with new connective tissues with minor infiltration of neutrophils. In the experimental group, numerous capillary blood vessels involving extension into gingival connective tissues and an irregular morphology were observed (Fig. 4A, B).

VEGF expression was seldom noted in the control group, whereas intensive expression was observed around dilated capillary blood vessels in connective tissues in the experimental group (Fig. 4C).

**Findings on immunohistochemical staining with anti-AGE antibody**

**Day 3 post-surgery**
A localization of AGEs in blood clots and the surrounding tissues was seldom observed in the control group, whereas it was noted in both blood clots and the capillary blood vessels in the surrounding connective tissues in the experimental group (Fig. 5A, B, C).

**Day 5 post-surgery**
In the control group, blood clots nearly disappeared and no localization of AGEs were observed in granulation tissues and the surrounding tissues. In the experimental group, a small number of few blood clots persisted and, as with Day 3 post-surgery, a localization of AGEs was noted both in blood clots and around capillary blood vessels in the surrounding connective tissues (Fig. 6A, B, C).
Day 7 post-surgery  
In the control group, no localization of AGEs was observed in gingival connective tissues. In the experimental group, a small number of blood clots persisted and a localization of AGEs was noted both in blood clots and around capillary blood vessels in the surrounding connective tissues (Fig. 7A, B, C).

DISCUSSION  
VEGF is secreted in not only a variety of normal cells, such as vascular smooth muscle and cardiac muscle cells, but also cancer cells. It selectively acts on vascular endothelial cells and has two properties: vascular growth or regeneration and promotion of vascular hyperpermeability.

The mechanisms of VEGF expression, including activation of the Ras signaling system, hypoxic stimulation, and estrogen stimulation, are known.

Furthermore, VEGF is closely involved in physiologically normal vascularization, as well as pathological vascularization under several conditions, such as in the presence of cancers and diabetic retinopathy, as demonstrated by the fact that it is secreted from cancer and inflammatory cells.

This study also demonstrated that VEGF was expressed in the surgical fields on Days 3 and 5 post-surgery in both experimental and control groups. This may be due to the spontaneous interruption of blood circulation in these fields during periodontal surgeries, resulting in a hypoxic state, which would have increased VEGF expression.

Diabetic retinopathy, which is one of the three major diabetic complications, commonly involves the basic pathophysiology: retinal blood vessels are impaired as a result of abnormal metabolism due to hyperglycemia, which induces vascular obstruction or hyperpermeability. In the early stage of diabetic retinopathy, capillary aneurysm and retinal bleeding as a result of damage to the wall of retina blood cells, as well as retinal edema, hard exudate, and vascular deformation due to vascular hyperpermeability are observed (preproliferative diabetic retinopathy).

The further obstruction of retinal blood vessels causes a hypoxic state called retinal ischemia, resulting in pathological vascularization in the retina or iris. This leads to the functional disability of serious visual impairment (proliferative diabetic retinopathy).

The impairment of endothelial cells and pericytes in small blood vessels of the retina underlies these pathologies. Growth factors such as VEGF have been strongly suggested to contribute to this impairment. In addition, if the hyperglycemic condition persists, AGEs will accumulate in the body. These AGEs, once recognized by the receptors (receptor for AGEs: RAGE) on vascular endothelial cells, increase the intracellular production of oxidative stress and accelerate the autocrine reaction of VEGF in vascular endothelial cells.

In the present study, VEGF expression was observed in connective tissues in the surgical fields in both experimental and control groups and, in the experimental group, a more intensive expression was noted specifically around capillary blood vessels on Days 3 and 5 post-surgery. On the other hand, AGEs were very rarely observed in the control group, while localized around capillary blood vessels in connective tissues in the experimental group throughout the experimental period.

In the case of diabetic retinopathy, AGEs reportedly play a role in selective pericyte loss, i.e., apoptosis, as well as to VEGF expression in the early stage of the pathology. As described above, the expression of VEGF observed in this experiment is attributed to surgery-induced ischemia, leading to
the occurrence of a hypoxic state in the surgical field. However, VEGF was more intensely expressed around capillary blood vessels in the experimental compared to the control group.

The reason for this finding may be that, likewise to the phenomenon seen in capillary blood vessels in diabetic retinopathy, AGEs present in the surgical field would have increased the production of vascular oxidative stress and, thereby, more intensively induced VEGF expression in vascular endothelial cells.

Furthermore, the present study employed the 8-week-old GK rats that were raised for 24 weeks and showed a persistent hyperglycemic condition with a blood glucose level of 200 mg/dL or above. Therefore, AGEs that had been accumulated in the body in the presence of a chronic hyperglycemic condition could have been more intensively localized in these rats.

On the other hand, more capillary blood vessels and capillary vasodilation were observed in the experimental group than in the control group; this was consistent with the result that highly-angiogenic VEGF was expressed in the corresponding region. Kono et al. conducted a similar experiment, and found histological pictures indicating the growth of capillary blood vessels, including sprouting and intussusception, as well as vascular hyperpermeability, such as vesicles of the cell membrane\(^25\).

On Day 7 post-surgery, VEGF expression was very rarely observed in the control group, whereas it was identified around capillary blood vessels in gingival connective tissues in the experimental group. The reason why the control group did not show VEGF expression is that the blood circulation had almost fully recovered in periodontal defects and, thereby, the hypoxic state was improved.

In the experimental group, the pathological growth of blood vessels still persisted on Day 7 post-surgery, resulting in the delayed recovery of blood circulation relative to the control group. This caused a hypoxic state due to poor circulation in the surgical fields, and would have then induced further expression of VEGF. Moreover, AGEs were very rarely observed in the control group but noted around capillary blood vessels in connective tissues throughout the experimental period in the experimental group. This suggests that, throughout this period, AGEs promoted the autocrine action of VEGF in vascular endothelial cells.

Based on these results, AGEs may increase the production of oxidative stress through AGEs-RAGE in periodontal defects prepared by periodontal surgery in the experimental group and, thereby, promote a more intensive expression of VEGF compared to the control group, leading to persistent vascularization and vascular hyperpermeability in the surgical fields.

The persistent expression of VEGF may cause the growth of pathological capillary blood vessels and a more severe hypoxic state in periodontal defects, which would further induce VEGF expression. The results of this study suggest that the contribution of VEGF to vascularization through these AGEs may affect the recovery of microcirculation during wound healing of artificial periodontal defects in the experimental group. Thus, if we can control VEGF expression by inhibiting AGEs-RAGE in periodontal tissues of diabetic patients, the success rate of periodontal surgeries, including regenerative therapy for periodontal tissues, would be improved.

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