ORIGINAL   ARTICLE

# Effects on Circulating VEGF Concentration on Periodontal Surgery in Diabetic Rats

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**SYNOPSIS**
Vascular endothelial growth factor (VEGF) has been recognized as closely related diabetic complications. In this study, we created periodontal defects in diabetic model rats and examined the effects of VEGF expressed in wound healing process on the circulating VEGF concentration.

In type 2 diabetic model (GK) and normal (SD) rats, periodontal defects were prepared in the bilateral upper molar regions. The VEGF expression in the periodontium 3, 5, and 7 days after surgery was examined by immunohistochemical staining. In addition, circulating blood was collected from the right atrium, and the plasma VEGF concentration was determined by ELISA.

VEGF was expressed more intensely around microvessels, and the plasma VEGF concentration was higher in GK rats than in the SD rats throughout the study.

These results suggest that, after periodontal surgery in diabetic rats, VEGF expressed in the periodontium affects the circulating VEGF concentration.

**Key words**: *circulating VEGF concentration, periodontal surgery, diabetes mellitus*

**INTRODUCTION**

The number of diabetic patients in Japan is estimated to be 7.2 million, and a total of about 22 million are considered to have, or be at a high risk of, diabetes[1]. Diabetes is classified into types 1 (insulin-dependent) and 2 (non-insulin- dependent), but type 2, which has an onset in middle to old age, is predominant, accounting for about 95% of all diabetic patients. Once it develops, diabetes causes various complications, which are classified into macro- and microvascular disorders[2,3]. Macrovascular disorders include cerebrovascular disturbances, such as cerebral infarction, and cardio- vascular disturbances, such as myocardial infarction. Microvascular disorders include retinopathy, nephropathy, and neuropathy. Vascular impairment is associated with the etiology of all these 5 diabetic complications[4]. In 2008, the Japan Diabetes Society proposed periodontal disease as the sixth complication of diabetes mellitus[5]. Diabetic patients frequently develop periodontal disease, and diabetes mellitus has been reported to be an important risk factor for the disease[3,6]. In addition, periodontal disease in diabetic patients tends to be severe and often requires periodontal surgery for its treatment.

Vascular endothelial growth factor (VEGF) is a glycoprotein that induces the proliferation /differentiation of vascular endothelial cells, enhancement of vascular permeability, and vasodilation and is an important angiogenic factor closely related to the process of angiogenesis[7]. VEGF is not only related to vasculogenesis in the embryonic period, structural development, and luteinization but also plays important roles in angiogenesis and enhancement of the vascular permeability under pathological conditions such as solid tumors, chronic rheumatoid arthritis, diabetic retinopathy, inflammation, and wound healing[8-12]. Since solid tumors excessively consume nutrition and oxygen, VEGF is expressed by vascular endothelial cells and induces pathological angiogenesis[13].

VEGF has also been reported to be expressed in chronic rheumatoid arthritis and diabetic retinopathy and to exacerbate of these diseases[14-16].

Presently, VEGF is clinically used as a blood test item. VEGF is indispensable for the angiogenesis necessary for the growth and metastasis of tumors. Therefore, the circulating plasma VEGF concentration has been reported to be significantly higher in patients with malignant neoplasms than in healthy controls[17-19]. It has also been reported that, in chronic rheumatoid arthritis patients, the VEGF concentration in the synovial fluid of the knee was 10-fold higher than that in plasma, and that the serum VEGF concentration was markedly higher in rheumatoid arthritis patients than in healthy controls[20-22]. There is also a report that the serum VEGF concentration was significantly higher in diabetic patients than in healthy individuals[23]. Moreover, the serum VEGF concentration has been reported to be significantly higher in diabetic patients with than in those without retinopathy[24,25]. Thus, there have been a number of reports that VEGF expressed at lesions and in disease states affects the circulating VEGF concentration.

In our previous studies involving type 2 diabetic model rats, we observed that VEGF was expressed more intensely around microvessels at sites of wound healing after periodontal surgery compared to normal rats and impairs healing by pathological angiogenesis[26-28].

However, it is not clear that VEGF expressed in the periodontium by periodontal surgery effects whole body through peripheral blood vessels in diabetes patients.

In this study, we created experimental defects in the periodontium of type 2 diabetic model rats and examined the effects of VEGF expressed at the defects during wound healing on the circulating VEGF concentration.

## MATERIALS AND METHODS

The present study was approved by the Committee for Animal Experiments of Osaka Dental University (Approval number: 14-01001) and conducted in accordance with the guidelines for animal experimentation.

### 1. Experimental materials

Twenty each of male Goto-Kakizaki (GK) rats (body weight 180-210 g; experimental group), an animal model with type 2 diabetes, and Sprague Dawley (SD) rats (body weight: 210-230 g; control group) were acquired from Shimizu Laboratory Supplies Co., Ltd. at the age of 8 weeks and raised for 37 weeks[29].

The experimental group consisted of only rats that showed a fasting blood glucose level of 200 mg/dL or above in prior assays of blood collected through the caudal vein using a Nipro Stat Strip XP2 (NIPRO, Osaka, Japan). Table 1 shows the body weight and blood glucose level in both groups.

## 2. Experimental methods

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

The rats were anesthetized by the inhalation of isoflurane (Forane®, Abbott, North Chicago, 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 a supine position with their mouths open. Surgery was performed according to the following procedures to observe the periodontium including the central roots on the palatal side of the 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 mesiopalatal surface of the maxillary first molar to the mesiopalatal angle of the third molar. 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 mesiopalatal angle to the third molar mesiopalatal angle, the alveolar bone, periodontal membrane, and tooth root surface (cementum and dentin) were curetted away using a Mini Five Gracey Curette (#7/8, Hu-Friedy, Chicago, IL, U.S.A.) and double-ended chisel (Ochsenbein #3, 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 single-suture was performed using a 6-0 absorbable thread (Biosorb C®, Alcon, Hünenberg, Switzerland) in the mesial area of the first molar (Fig. 1). Hemostasis was achieved by compressing the surgical site for about 2 minutes using a cotton ball saturated with physiologic saline.

The experimental periods were 3, 5, and 7 days after surgery[30-32]. Five rats each were employed for the experimental and control groups in each period. Baseline values of the circulating VEGF concentration were measured in 5 preoperated animals each in the experimental and control groups.

## 3. 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 via the left atrium, and 10% neutral formaldehyde solution (Nacalai Tesque, Kyoto, Japan) was perfused for fixation. Tissue containing the experimental dental root was excised en bloc, immersed in fresh fixation solution at 4°C for 3 days, and decalcified in a rapid decalcification solution (K-CX, Falma, Tokyo, Japan) at 4°C for 24 hours. After removing excess tissues, the decalcified samples were split in the buccopalatal direction on the mesial side of the mesiopalatal root of the first molar. The split samples were washed with 0.1 M phosphate buffer solution (PBS; pH 7.2) at 4°C and embedded in paraffin by the routine method. Then, 5-μm serial longitudinal sections allowing observation of the mesiopalatal root of the first molar nearly to the root apex were prepared. The thin sections of both the experimental and control groups were stained immunohistochemically using anti-VEGF monoclonal antibody (sc-7269, Santa Cruz, Biotechnology, California, USA)[33]. For this staining, the sections were deparaffinized and reacted with pepsin adjusted to 0.4% using 0.01 mol/L HCl for 30 minutes to activate antigens. After the inactivation of endoge-

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nous peroxidase with 0.3% $H_2O_2$, the sections were treated overnight with anti-VEGF monoclonal antibody diluted 50-fold with PBS at 4°C and colored using Dako EnVision+ System (Dako-Cytomation, Glostrup, Denmark) and 3,3-diaminobenzidine-tetrachloride (DAB, DakoCytomation, Glostrup, Denmark) [34]. Subsequently, the sections were treated with hematoxylin for nuclear staining, dehydrated, enclosed, and examined under an All-in-One Fluorescence Microscope (BZ9000, KEYENCE, Tokyo, Japan).

## 4. Sampling of circulating blood and measurement of the VEGF concentration

The rats of both control and experimental groups were euthanized with an overdose of Somuno pentil injection[®] after each period. Thoracotomy was performed immediately, and about 7 mL of circulating blood was collected from the right atrium using a 10mL syringe with a 22G injection needle (Terumo, Tokyo, Japan) in vacuum sampling tubes (Venoject[®] II, Terumo, Tokyo, Japan). The sampled blood was centrifuged at 1,000 rpm for 20 minutes. The plasma fraction was collected, divided in microtubes at 50 µL/tube, and stored by freezing at -20°C.

The circulating VEGF concentration was measured using Rat VEGF (Quantikine [®] ELISA, R&D Systems, Minneapolis, MN, USA). Firstly, an analytical diluent was added at 50 µL/well. Next, the plasma fraction was thawed at room temperature and added at 50 µL/well. Then, the microplate was incubated at room temperature for 2 hours on a horizontal orbital microplate shaker (500±50 rpm). Each well was aspirated and washed 5 times with a washing solution prepared by diluting a washing buffer 25-fold. Thereafter, 100 µL of rat VEGF conjugate was added to each well and incubated again at room temperature for 1 hour on a microplate shaker (500±50 rpm). Then, the plate was washed again 5 times with the washing solution and, after adding a substrate solution at 100 µL/well, reacted over 30 minutes in the dark. Then, a stop solution (diluted HCl) was placed at 100 µL/well, and the VEGF concentration was determined by measuring the absorbance using a microplate reader (Spectra Max Pro, Molecular Device, USA) at 450 nm and a wavelength correction of 540 or 570 nm.

## 5. Statistical analysis

The body weight and blood glucose level were compared between the experimental and control groups by the unpaired t-test. The circulating VEGF concentration was compared between the two groups after each experimental period by the Mann-Whitney U-test. The circulating VEGF concentrations in each group after various experimental periods were compared by the Kruskal-Wallis H-test.

**Table 1**  Body weight and blood glucose level of the rats

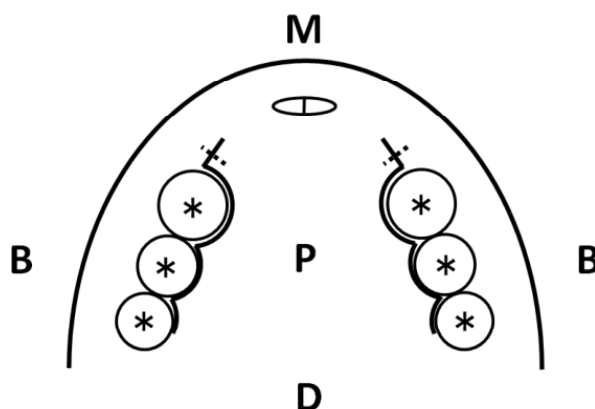|  | Body weight (g) | Blood glucose level (mg/dL) |
|---|---|---|
| Control group | 637.8±81.33 | 149.6±22.75 |
| Experimental group | 445.1±26.98 | 287.0±21.33 |

Mean±SD, p<0.01

**Fig. 1**  Schema of a rat's maxilla
（Full line）：Incision
（Dotted line）：Suture

## RESULTS

### 1. Body weight and blood glucose level

The mean body weight was significantly lower in the experimental group than in the control group. The mean blood glucose level was significantly higher in the experimental group than in the control group (Table 1) [24].

### 2. Findings on immunohistochemical staining with anti-VEGF antibody
#### 1) Day 3 post-surgery

Blood clots consisting primarily of fibrin were observed immediately below the returned gingival flap in both the experimental and control groups. VEGF was expressed intensely in the blood clots and around blood vessels of gingival connective tissue adjacent to the surgical site (Fig. 2 A,B,C,D).

#### 2) Day 5 post-surgery

In the experimental group, VEGF was expressed intensely in the blood clots and around blood vessels of the subepithelial gingival connective tissue. In the control group, few blood clots were observed, and the VEGF expression in connective tissue was weaker than in the experimental group (Fig. 3 A,B,C,D).

#### 3) Day 7 post-surgery

Blood clots disappeared in both the experimental and control groups. In the experimental group, VEGF was expressed around blood vessels of subepithelial connective tissue. In the control group, VEGF expression was not noted (Fig. 4 A,B,C,D).

### 3. Circulating VEGF concentration
#### 1) Before surgery (preoperated state)

The circulating VEGF concentration was significantly higher in the experimental group than in the control group (P<0.05) (Fig. 5).

#### 2) Days 3, 5, and 7 post-surgery

The circulating VEGF concentration was significantly higher in the experimental group than in the control group during experimental periods (P<0.05)(Fig.6,7,8).

#### 3) Chronological changes in the circulating VEGF concentration

Compared with the preoperative baseline value, the circulating VEGF concentration was significantly higher on Days 3 and 5 post-surgery in both groups. On Day 7, no significant difference compared with the baseline value was observed in either group (Fig. 9,10).
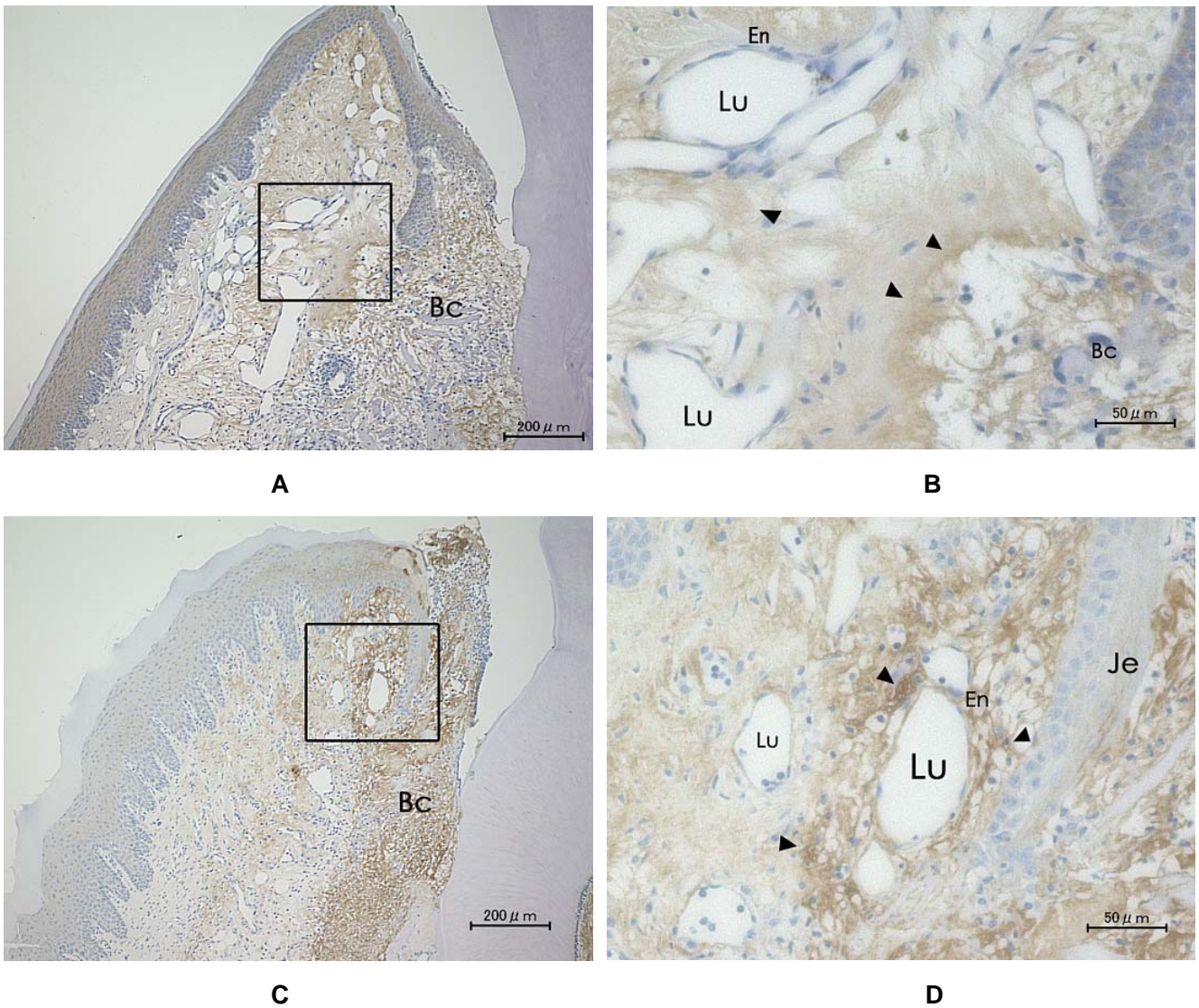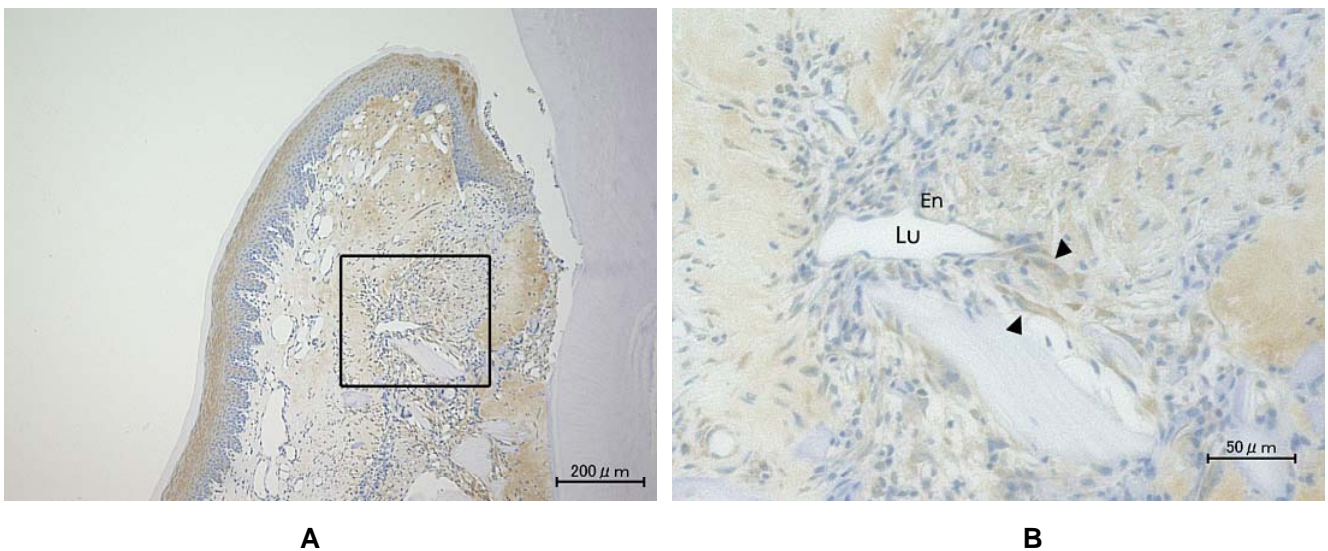
A



B



C



D

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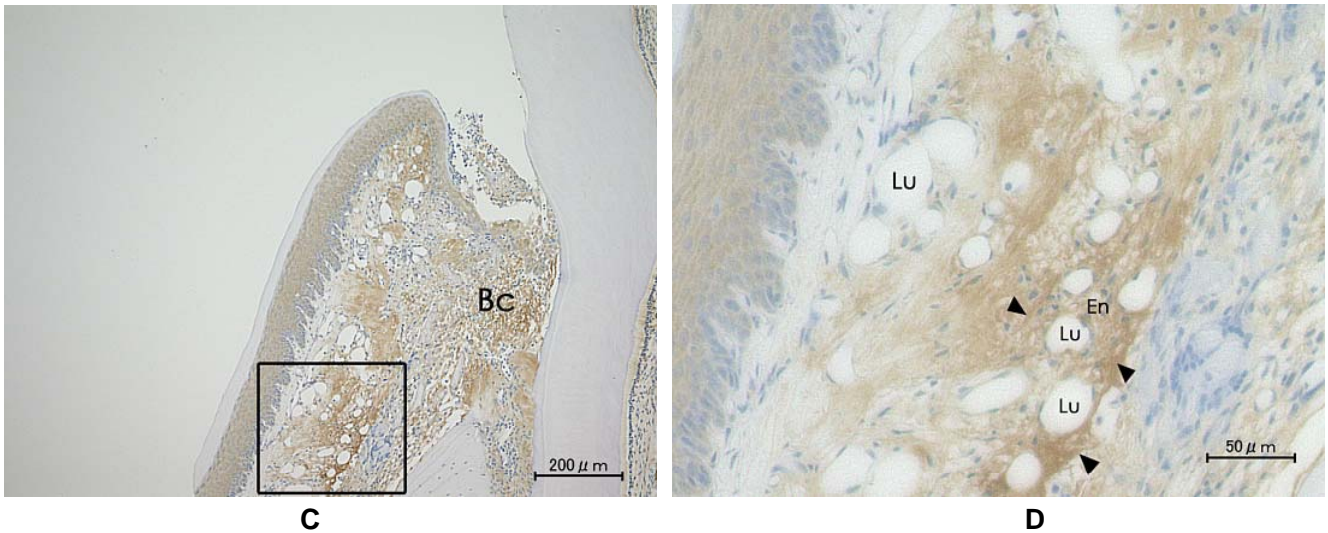　　　　(▲): Expression of VEGF



A



B

C



D

**Fig. 3**   Immunohistochemical staining with anti-VEGF antibody on day 5 post-surgery
 A. Control group   ×100
 B. Control group   higher magnification of A   ×400
 C. Experimental group   ×100
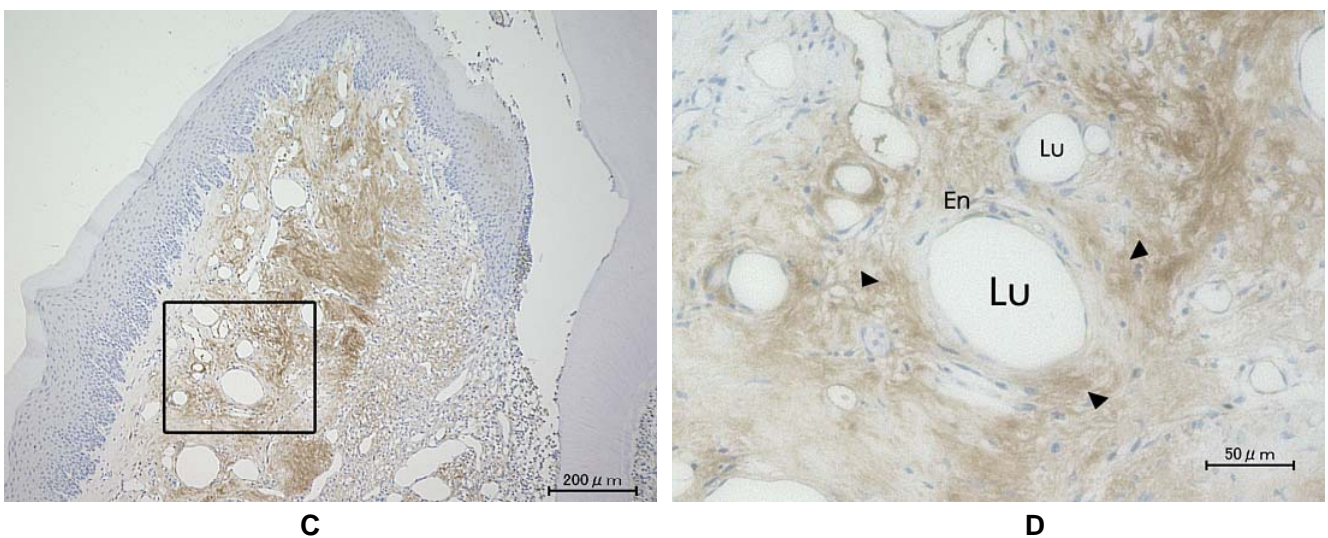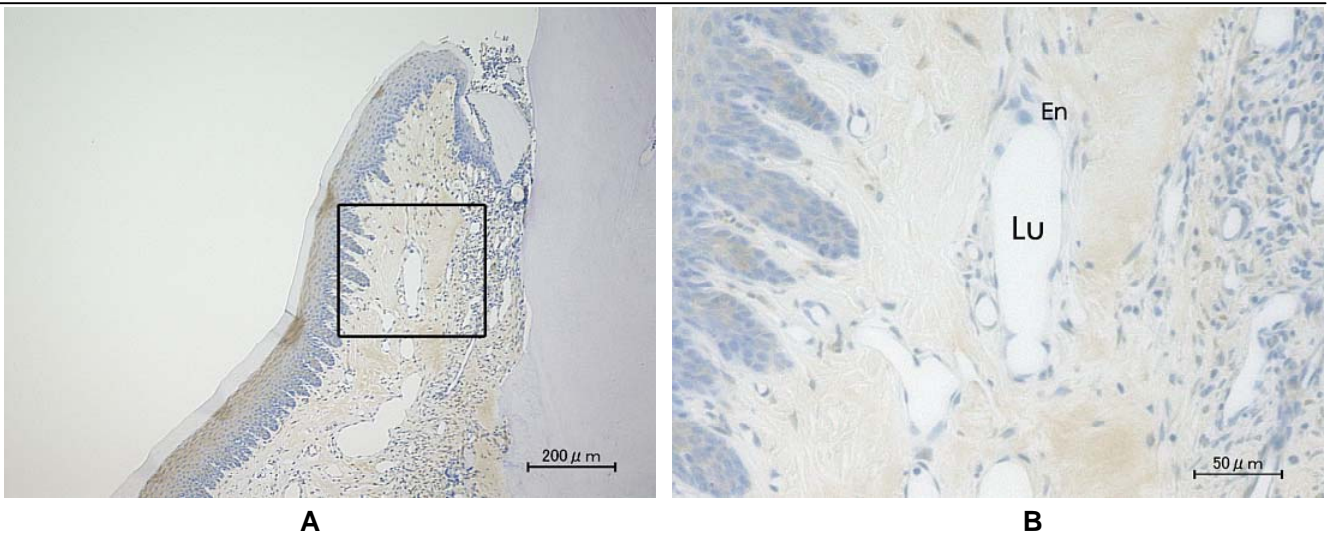 D. Experimental group   higher magnification of C   ×400   （▲）: Expression of VEGF



A



B



C



D

**Fig. 4**   Immunohistochemical staining with anti-VEGF antibody on day 7 post-surgery
 A. Control group   ×100
 B. Control group   higher magnification of A   ×400
 C. Experimental group   ×100
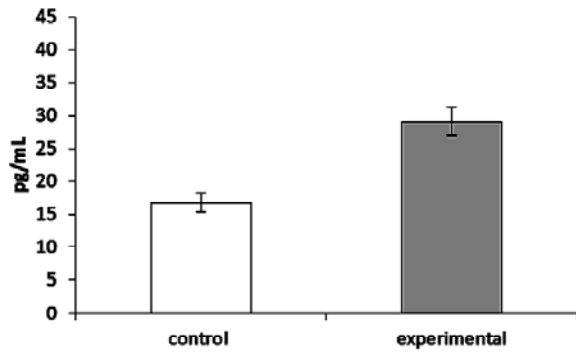 D. Experimental group   higher magnification of C   ×400   （▲）: Expression of VEGF

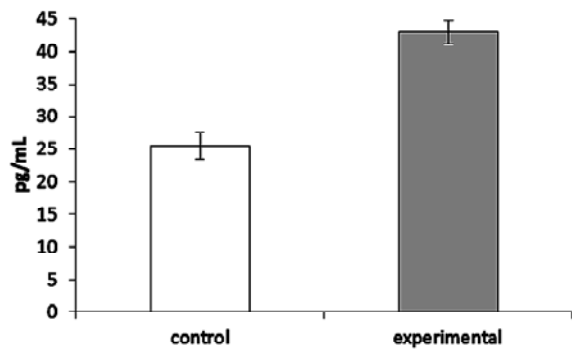**Fig.5**  Circulating VEGF concentration (preoperated state)



**Fig.6**  Circulating VEGF concentration (Day 3 post-surgery)
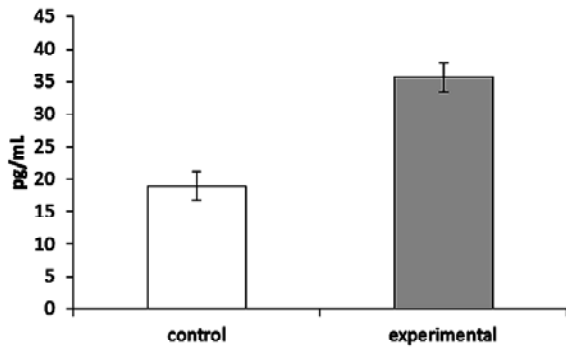


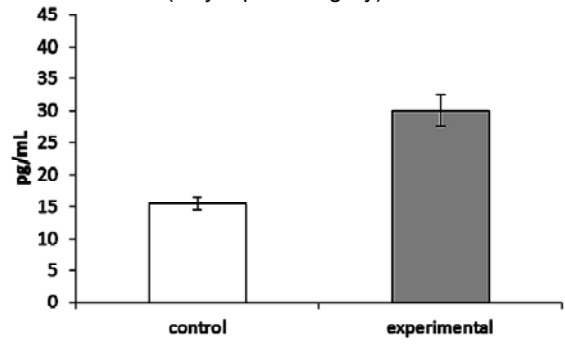**Fig.7**  Circulating VEGF concentration (Day 5 post-surgery)



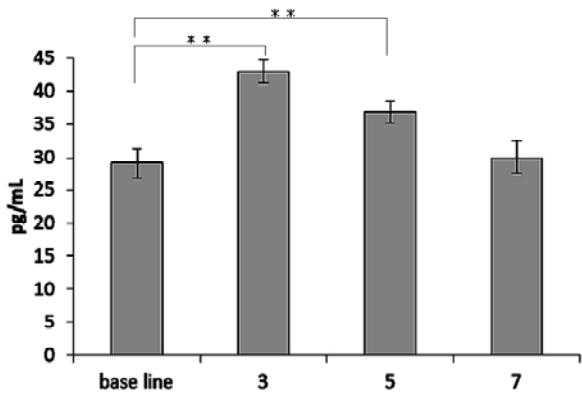**Fig.8**  Circulating VEGF concentration (Day 7 post-surgery)



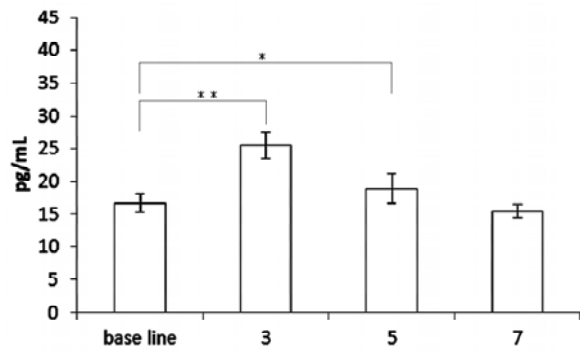**Fig.9**  Chronological changes in the circulating VEGF concentration (experimental) (＊＊P<0.01)



**Fig.10**  Chronological changes in the circulating VEGF concentration (control) (＊＊P<0.01) (＊P<0.05)

## DISCUSSION

VEGF is a growth factor discovered in 1989 by Ferrara *et al.* that specifically acts on vascular endothelial cells and characteristically promotes vascularization and vascular hyperpermeability[7,35].

VEGF expression is known to require activation of the Ras signaling system, keratinocytes, estrogen stimulation, and hypoxic stimulation by a hypoxia inducible factor[12,36].

In addition, VEGF is not only contributes to vasculogenesis in the embryonic period and angiogenesis of normal tissues such as smooth muscle, cardiac muscle, and liver but is also secreted at solid cancer and inflammatory tissues and is closely involved in

pathological angiogenesis in many diseases including cancer, chronic rheumatoid arthritis, and diabetic retinopahy[37-42].

Presently, VEGF is used clinically as a blood test item. The circulating VEGF concentration has been reported to be significantly higher in patients with various diseases in which VEGF is associated with pathological angiogenesis than in healthy individuals[18,19,21-23].

In this study, significant differences were noted in the body weight and blood glucose level between the experimental and control groups. GK rat is non-obesity model rat. Moreover, it is known that a weight less is caused when diabetes mellitus makes it serious. This demonstrated that the GK rats used as the experimental group had truly developed diabetes. At the baseline, the plasma VEGF concentration was significantly higher in the diabetic model rats than in healthy rats. Kakizawa *et al.* compared the serum VEGF concentration between diabetic patients and healthy controls and reported that it was significantly higher in the patients[23]. It has also been reported that the VEGF production in the culture medium of vascular endothelial cells increased with the glucose concentration[43,44]. Similar results were observed in the diabetic rats used in this study.

In this study, the plasma VEGF concentration reached a peak on Day 3 post-surgery in both groups, decreased on Day 5, and showed no significant difference compared with the preoperative level on Day 7. In the periodontium, VEGF was expressed in blood clots and around subepithelial blood vessels on Day 3 after surgery. On Day 5, VEGF expression similar to that on Day 3 was observed in the experimental group, but VEGF expression was not observed on connective tissue close to the epithelium in the control group. On Day 7, VEGF was expressed in the experimental group but not in the control group.

In both groups, VEGF expressed in the periodontium after periodontal surgery affected the circulating VEGF concentration through peripheral blood vessels, but, in the control group, the significant difference in the plasma VEGF concentration compared with the preoperative level disappeared on Day 7 with the disappearance of VEGF expression in the periodontium. However, in the experimental group, VEGF was still expressed in the periodontium on Day 7, but the plasma VEGF concentration decreased to a level not significantly different compared with the preoperative level.

In diabetic retinopathy, retinal vessels are damaged by abnormal metabolism due to hyperglycemia, hypoxia is induced by the occlusion of retinal vessels, and VEGF expressed at the site of vascular damage. Thus, it has been established that VEGF promotes pathological retinal angiogenesis[15,16,41,42]. Moreover, Takayama *et al.* reported that the serum VEGF concentration was significantly higher in patients with diabetic retinopathy than without. They suggested that VEGF expressed in the retina affect the serum VEGF concentration through peripheral blood vessels[24,25]. Also, it has been reported that diabetic rats show marked VEGF expression at the site of surgery and associate pathological angiogenesis and vascular hyperpermeability which affect the delay in wound healing after periodontal surgery compared with normal rats[26-28]. In this study, VEGF induced by hypoxia caused by periodontal surgery in the periodontium is considered to have increased the circulating VEGF concentration through peripheral blood vessels. However, in both groups, the plasma VEGF concentration decreased almost to the preoperative level on Day 7, when VEGF expression still persisted in the periodontium in the ex-

perimental group. It might be that the VEGF expression in the periodontium persisted on Day 7 in diabetic rats, although at a level insufficient to affect the circulating VEGF concentration.

VEGF expression level in periodontium can not be measured by immunohistochemical staining. Therefore, we will observe VEGF expression level in periodontium in future.

Through the entire experimental period, the plasma VEGF concentration differed significantly between the two groups, always being higher in the experimental group than in the control group. As mentioned above, there is a report that the serum VEGF concentration was significantly higher in diabetic patients than in healthy individuals[23]. In this study, the baseline difference in the plasma VEGF concentration between the experimental and control groups persisted until after periodontal surgery to the end of the experiment. This suggests that, in diabetic rats, periodontal surgery affects the plasma VEGF concentration in the experimental group more intense than in the control group.

In this study, the plasma VEGF concentration in diabetic rats was higher than in normal rats before periodontal surgery. And the concentration in diabetic rats at Day 3 and Day 5 after surgery was higher than baseline, but backed to the baseline at Day 7. This suggests that, if periodontal surgery is performed for the treatment of periodontal disease in diabetic patients, VEGF expressed in the periodontium markedly elevates the circulating VEGF concentration via peripheral vessels until 5 day after periodontal surgery and may cause or exacerbate diabetic complications in other organs such as retinopathy and nephropathy. Furthermore, until Day 7 after surgery, when the plasma VEGF concentration recovers to the preoperative level, careful monitoring for systemic effects of the surgical procedure is considered necessary.

## ACKNOWLEDGMENTS

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