Expression of vascular endothelial growth factor on periodontal early wound healing in model rats with type 2 diabetes mellitus

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Vascular Endothelial Growth Factor (VEGF) plays a significant role in microangiopathy. This study aimed to observe VEGF expression immunohistochemically in the early healing stage of periodontal defects in Goto-Kakizaki (GK) model rats with type 2 diabetes and clarify the relationship between VEGF and microangiopathy in the healing process. We surgically prepared periodontal defects in the maxillary molars of male GK and Sprague Dawley (SD) rats. The animals were euthanized 3 and 7 days after surgery. Periodontal samples were stained immunohistochemically with anti-VEGF monoclonal antibodies. Other samples were observed with a transmission electron microscope. We found localization of VEGF in the periodontal defect region 3 days after surgery in both groups. VEGF was strongly localized around the vessels of the experimental group and active formation of new vessels was confirmed. Although VEGF was rarely localized around the vessels in the control group 7 days after surgery, localization occurred in the experimental group around capillaries in the periodontal defect region and there was formation of new vessels. VEGF expression in the experimental group was significantly greater than that in the controls, resulting in continuous formation of new, immature blood vessels. These results suggest that microcirculation recovery is delayed in the healing of periodontal defects. (J Osaka Dent Univ 2012; 46: 237-243)

Key words: Vascular endothelial growth factor, Periodontal wound healing, Type 2 diabetes mellitus

INTRODUCTION

Diabetes mellitus has been reported to be an important risk factor for periodontal disease, which is now regarded as the sixth most common diabetic complication following retinopathy, nephropathy, neuropathy, microangiopathy and macroangiopathy. All of these diabetic complications are caused by angiopathy (vascular impairments). The high incidence of periodontal disease in diabetic patients may be partly explained by the possible presence of microcirculatory impairments in the periodontium. 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 angiogenesis, which occurs, for example, in the course of wound healing. VEGF is also known to be involved in normal vascularization, and plays an important role in pathological vascularization or conditions associated with vascular hyperpermeability, such as malignancies and diabetic retinopathy.

In this study, to elucidate the relationship between VEGF and microangiopathy in wound healing in diabetic patients, we created periodontal defects in the maxillary molars of GK rats. We then examined VEGF expression in the early stages of the healing process and studied the ultrastructure of newly formed microvessels.
MATERIALS AND METHODS

Experimental animals
This study was approved by the Committee for Animal Experiments of Osaka Dental University (Approval number: 10–03002) and was conducted in accordance with the Guidelines for Animal Experimentation. Twelve male GK rats, which are an animal model with type 2 diabetes, were used for the experimental group, and 12 Sprague Dawley (SD) rats aged 8 weeks were used as controls in this study. The experimental group consisted of only rats that showed a fasting blood glucose level of 200 mg/dL or above in measurements collected through the caudal vein using a Nipro Free Style FlashTM (Nipro, Osaka, Japan).

Methods

Surgical procedure
Preoperatively, fasting glucose levels were again measured in both the experimental and control groups. The rats were anesthetized by the inhalation of isoflurane (Forane®, Abbott, North Chicago, USA), injected intraperitoneally with 0.3 mg/kg of pentobar-

Fig. 1 Immunohistochemical staining with anti-VEGF antibody (×100). A: Three days post-surgery in the control group, B: Three days post-surgery in the experimental group, C: Seven days post-surgery in the control group, D: Seven days post-surgery in the experimental group.
bital sodium (Somuno pentil injection\(^{1}\), Kyoritsu Seiyaku, Tokyo, Japan), and fixed in the supine position with their mouths open. Surgery was performed to observe the periodontium, including the central roots on the palatal side of the bilateral maxillary first molars, by the following procedures. An internal bevel incision was made using a slit-knife (Alcon, Hünenberg, Switzerland) in the gingival crevice from the center of the mesial palatal side of the surface adjacent to the maxillary first molar to the mesial palatal angle of the second molar. A full-thickness flap consisting of the mucosal epithelium, lamina propria, and periosteum was prepared in the incised area using a dental excavator (Hu-Friedy, Chicago, USA).

The alveolar bone, periodontal membrane, and cementum were curetted away from the mesial palatal angle of the maxillary first molar to the mesial palatal angle of the second molar using a Mini Five Gracey Curette (#7/8, Hu-Friedy, Chicago, USA) to create an artificial periodontal defect. Surgical fields were washed two times with physiological saline. The full-thickness gingival flap was then returned to its original position (Fig. 1). On days 3 and 7,\(^{1}\) six each of the GK and SD rats were euthanized with an overdose of Somuno pentil injection\(^{1}\) and the samples were collected.

**Preparation of samples for immunohistochemistry**

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. The tissues around the defect, including the 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 samples were cut in the buccal to palatal direction at the mesial side of the central palatal root of the first molar, after removing the excess tissue. Each sample was washed independently using 0.1 M phosphate buffer solution (PBS; pH 7.2) at 4°C and embedded in paraffin by conventional methods.

Serial sections with a thickness of 5 μm were then prepared over a range allowing observation of the central palatal root of the first molar. Immunohistochemical staining was performed with an anti-VEGF monoclonal antibody (sc-7269, Santa Cruz Biotechnology, California, USA) in the sections of both groups.\(^{9}\) For this staining, 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 the antigens. After inactivating endogenous peroxidase in 0.3% H\(_2\)O\(_2\), sections were reacted with anti-VEGF monoclonal antibody diluted 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\(^{TM}\) system (DakoCytomation)\(^{10}\). Subsequently, sections were subjected to nuclear staining with hematoxylin, dehydrated, enclosed, and observed using an All-in-One Fluorescence Microscope (BZ 9000, Keyence Japan, Tokyo, Japan).

The sections for both groups were subjected to immunohistochemical staining with an anti-AGEs monoclonal antibody (KH 001–02, TransGenic, Kumamoto, Japan).\(^{11}\) For this staining, sections were deparaffinized and then reacted with a 0.4% solution of pepsin in 0.01 N HCL at 37°C for 30 minutes to activate the antigens. After inactivating endogenous peroxidase in 0.3% H\(_2\)O\(_2\), the sections were reacted with anti-AGEs monoclonal antibody diluted 500 fold with PBS at 37°C for 60 minutes, and then colored with 3,3-diaminobenzidine-tetrahydrochloride using the EnVision\(^{TM}\) system. Sections were then subjected to nuclear staining with hematoxylin, dehydrated, enclosed and observed using the All-in-One Fluorescence Microscope.

**Transmission electron microscopy sample preparation**

Immediately after euthanasia, the chest was incised and a catheter was inserted into the ascending aorta, through which 2.5% glutaraldehyde (0.05 mol/L phosphate-buffer, pH 7.2) was perfused for fixation. The root and surrounding tissues were collected, immersed in fresh fixation solution at 4°C for three days, and then decalcified in decalcifying solution (hydrochloric acid-buffered 5% ethylenediamine-
tetraacetic acid-4 Na, Kishida Chemical, Tokyo, Japan) at 4°C for 1 month. The decalcified sample was sectioned in the buccal to palatal direction at the mesial side of the central palatal root of the first molar palatal central root after removing excess tissue. Each sample was subjected to postfixation with 2% osmic acid and was then dehydrated and embedded in Epon 812 (Nissin, Tokyo, Japan). Ultra-thin sections with a thickness of 1 μm were prepared using an Ultramicrotome (MT 7000, Leica Biosystems, Wetzlar, Germany), double-stained with uranyl acetate and lead citrate, and observed by TEM (H-7100, Hitachi, Tokyo, Japan).

RESULTS

Findings on immunohistochemical staining with the anti-VEGF antibody

Three days post-surgery

Blood clots were observed in the periodontal defects that consisted predominantly of fibrins, including neutrophils, in both the experimental and control groups. Capillary neovascularization was noted in the gingival connective tissues around these blood clots. More capillary blood vessels were observed in the experimental group than in the controls. Although VEGF expression was identified in the connective tissues around the blood clots in both groups, it was more intense in the experimental group (Figs. 1 A and B).

Seven days post-surgery

The blood clots had nearly disappeared 7 days after surgery in both groups, and the periodontal defects were nearly filled with new connective tissue that had minor infiltration of neutrophils. Numerous capillary blood vessels were observed in the experimental group that involved extension into the gingival connective tissue. Although VEGF expression was seldom noted in the control group, intensive expression was observed around capillary blood vessels in the connective tissue of the experimental group (Figs. 1 C and D).

TEM findings

Three days post-surgery

Capillary blood vessels were seen in the connective tissue around blood clots in the control group with an oval lumen lined by endothelial cells containing vesicles (Fig. 2 A). In contrast, capillary blood vessels with an irregular lumen were observed in the connective tissue around blood clots in the experimental group (Fig. 2 B).

Seven days post-surgery

Capillary blood vessels with perfectly-round lumens were observed in the periodontal defect and surrounding connective tissue in the control group (Fig. 3 A). In contrast, many capillary blood vessels with a flattened lumen were noted in the periodontal defect and surrounding connective tissue in the experimen-

Fig. 2 Transmission electron microscopy (TEM) three days post-surgery. A: Capillary blood vessels in the control group (→ Endothelial cells containing vesicles). B: Capillary blood vessels with an irregular lumen in the experimental group (EN: Endothelial cells, LU: Vascular lumen).
tal group (Fig. 3 B). Capillary proliferation was still active in new connective tissue and endothelial sprouting was observed (Fig. 3 C).

DISCUSSION

VEGF is secreted not only in a variety of normal cells, such as vascular smooth muscle and cardiac muscle cells, but also in cancer cells. It selectively acts on vascular endothelial cells and has two properties: vascular growth as well as regeneration and promotion of vascular hyperpermeability. The mechanisms of VEGF expression are known, including activation of the Ras signaling system, hypoxic stimulation, and estrogen stimulation. Furthermore, VEGF has been 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 its secretion from cancer and inflammatory cells. This study also demonstrated that VEGF was expressed in the surgical fields 3 days post-surgery in both groups. This may be due to the spontaneous interruption of blood circulation in these fields during periodontal surgery resulting in a hypoxic state, which would have increased VEGF expression. Furthermore, we speculate that the expression of VEGF is involved in the growth and development of new capillary blood vessels in the periodontal defect in the process of wound healing and in increased vascular permeability that promotes the supply of nutrients to cells that play a role in promoting wound healing.

Three days post-surgery, VEGF was localized in the periodontal defect region in both the experimental and control groups. It was localized more strongly around the capillaries of the experimental group than
in those of the controls. It has been suggested that advanced glycation end-products (AGEs) result from a metabolic abnormality of hyperglycemia and induce apoptosis (involved in pericyte loss) and VEGF gene expression during the early stages of diabetic retinopathy. Retinopathy is one of the three major complications of diabetes.\textsuperscript{15,16,21-23} We speculate that AGEs induced VEGF expression by increasing the generation of oxidative stress products in the experimental group, as is seen in retinal capillaries in diabetic retinopathy. TEM demonstrated active angiogenesis in the experimental group, in agreement with VEGF localization with potent angiogenic activity at the same site.

Seven days post-surgery, although VEGF was localized around capillaries in the periodontal defect area in the experimental group, but little or no VEGF was localized in the controls, where numerous capillary blood vessels were proliferating actively, some of which showed endothelial sprouting. These findings suggest that blood circulation recovery was delayed due to VEGF involvement even 7 days postsurgery in the experimental group, and suggested that the resulting oxygen deficiency induced further VEGF expression, thereby contributing to a delay in wound healing. However blood circulation had almost recovered in the control group. The results of this study suggest that VEGF expression was promoted in the surgically created periodontal defect in the experimental group and that the resulting delay in microcirculation recovery induced further VEGF expression, thereby adversely affecting wound healing.

REFERENCES
