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Lymphangiogenesis and NOS Localization in Healing Process after Tooth Extraction in Akita Mouse

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Abstract

Type I diabetes, an autoimmune disease, induces insulin deficiency, which then disrupts vascular endothelial cell function, affecting blood and lymphatic vessels. Nitric oxide (NO) is an immune-induced destructive mediator in type I diabetes, and inhibition of its production promotes arteriosclerosis. In this study, lymphangiogenesis and expression of NO synthase (NOS) during the healing process after tooth extraction were investigated immunohistochemically in control (C57BL) and Akita mice as a diabetes model. Between 1, 4, and 10 days after extraction, expression of NOS, vascular endothelial growth factor-C (VEGF-C), VEGF receptor-3 (VEGFR-3), and von Willebrand factor was strongest during the granulation tissue phase. This suggests that severe inflammation triggers regulation of NOS and these other angiogenic and lymphangiogenic factors. During the callus phase, a few days after extraction, induced osteoblasts were positive for VEGF-C and VEGFR-3 in both the control and Akita mice, suggesting that bone formation is active in this period. Bone formation in the Akita group exceeded that in the controls. Bone tissue formation was disrupted under hyperglycemic conditions, however, suggesting that such activity would be insufficient to produce new bone.

Key words: Anatomy — Histology — Morphology — Bone — Nitric oxide synthase

Introduction

Type I diabetes is considered to be an autoimmune disease[5]. Nitric oxide (NO) has been reported to be involved in the development of a range of autoimmune diseases,

including type I diabetes, where it acts as an immune-induced destructive mediator[14]. Nitric oxide synthase (NOS) is classified as neuronal (nNOS), inducible (iNOS), or endothelial (eNOS). The production and release of nNOS are inhibited in diabetes, disrupting its function[8]. Inducible NOS is produced by activation of macrophages and monocytes in the Islets of Langerhans. This then stimulates production of NO, with subsequent destruction and impaired function of β cells[14]. In small amounts, NO plays a number of important roles, including in insulin secretion. Inducible NOS, however, produces NO in large amounts, directly damaging β cells[16]. Endothelial NOS is expressed in vascular endothelial cells. A reduction in eNOS production in diabetes impairs dilatation of capillary blood vessels[26]. Thus, there is a close relationship between NOS and the development of diabetes, particularly with regard to the dynamics of blood and lymphatic vessels. Recently, this group reported expression of NOS and reactive oxygen synthase (Nox) in intraoral tissue and bone[2,11,15]. However, another investigation of NOS in the oral tissue of a diabetic model demonstrated change in iNOS localization between normal dental pulp and pulpitis only[7], with none observed in expression of nNOS, iNOS, or eNOS in extraction sockets.

The histological healing process in extraction sockets is divided into 4 phases: the blood clot phase, from the 0–2nd day after extraction; the granulation tissue phase, from the 3–5th day; the callus phase, from the 6–14th day; and the healing phase, from the 15–20th day[3]. Change over time during this process has been reported in various animal species[1], including humans[6]. Many studies have investigated angiogenesis and the vascular dynamics involved in healing after tooth extraction in normal animals and diabetes models[1]. To our knowledge, however, none have investigated the timing or distribution of lymphangiogenesis or the presence or absence of lymphatic vessels in extraction sockets, as no reliable marker of lymphatic vascular dynamics in intraoral tissue was available. In one recent study by this group focusing on receptors, however, it was possible to accurately identify lymphatic vessels by targeting the combination of vascular endothelial growth factor-C (VEGF-C), VEGF-D, VEGF receptor-3 (VEGFR-3), and lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1)[25].

Diabetes model studies have commonly used drug-treated experimental animals. Recently, however, a new strain of mutant, the Akita mouse, has become available, and is now widely used as a mouse model of type I diabetes, as they show increased blood glucose levels, as specifically observed in type I diabetes. This type of mouse also shows the delayed oral tissue development and dysplasia associated with free radicals and active oxygen species[28]. However, to our knowledge, no study to date has investigated the interaction between change in NO after tooth extraction and angiogenesis or lymphangiogenesis in a diabetes model. Reports on how diabetes affects bone formation differ: it has been reported to affect bone formation due to impairment of osteoblasts[6]; diabetic hyperglycemia induced osteogenesis[21]; and no influence of diabetes was observed on bone healing in extraction sockets[23]. This indicates that Akita mice may offer a useful means of clarifying the effect of diabetes on bone formation.

The purpose of this study was to investigate the healing process after tooth extraction in normal and diabetic mice, focusing on lymphangiogenesis and expression of NOS. In addition, to distinguish vascular and lymphatic endothelial cells, the involvement of fibroblasts, endothelial cells, and osteoblasts in the healing of extraction sockets was immunohistochemically investigated in serial sections using von Willebrand factor (vWF), which is specific to vascular endothelial cells.

Materials and Methods

This study was performed following the animal experiment guidelines of Ohu University (Approval Number: H26-10). Twenty-four 12-week-old C57BL/6J mice (SLC, Shizuoka,

Japan) and 37 Akita/Slc mice (SLC) were used. The mandible was examined at 1, 4, and 10 days after extraction of the lower first molar.

The C57BL/6J and Akita/Slc mice were intraperitoneally anesthetized with Somnopentyl. Next, they were fixed by perfusion with saline and then 4% paraformaldehyde using a peristaltic pump. After fixation, the mandible was excised and fixed by immersion in the same fixative overnight. The mandible was then demineralized with 10% EDTA at 4°C. The demineralized sample was dehydrated with alcohol, embedded in paraffin, and 10-μm-thick serial sections prepared using a microtome.

The sections were deparaffinized with alcohol and treated with 0.3% H_2O_2-containing methanol solution for 10 min to inactivate endogenous peroxidase. The sections were then blocked with 10% normal rabbit serum and 10% normal goat serum (Vector Laboratories, California, USA) for 1 hr and reacted with the following 6 primary antibodies for 10 hr: rabbit anti-human nNOS (Invitrogen, California, USA), rabbit anti-human NOS2 (Santa Cruz, Texas, USA), rabbit anti-human NOS3 (Santa Cruz), goat anti-human VEGF-C (R&D Systems, Minnesota, USA), rabbit anti-human VEGFR-3 (Acris Antibodies GmbH, Herford, Germany), and factor VIII-related antigen/vWF Ab-1 (Thermo Scientific, Massachusetts, USA). The sections were then reacted with the following secondary antibodies: biotinylated anti-goat and biotinylated anti-rabbit antibodies (Vector Laboratories) for 1 hr, followed by a 1-hr reaction using the ABC method (Vector Laboratories). Color was developed using 0.3% H_2O_2-containing 0.005% 3.3-diaminobenzidinetetrahydrochloride solution (0.05 M Tris-HCI buffer, pH 7.6). The nuclei were stained with 5% methyl green (Muto Chemical, Tokyo, Japan) for immunohistochemical analysis. The preparations were observed under a light microscope. For washing sections and diluting antisera, 0.05 M Tris-HCI buffer (pH 7.6) was used. In the statistical analysis, the percentages and SD levels of endothelial cells positive for nNOS, iNOS, eNOS, VEGF-C, VEGFR-3, or vWF among the total numbers of blood and lymphatic vascular endothelial cells in 160×220 μm^2 were calculated at 1, 4, and 10 days after extraction and analyzed using Friedman's χ^2 r-test.

Results

1. Reactions during healing process after tooth extraction in control and Akita mice

1) Day 1 after tooth extraction

In the control group, fibroblasts had formed a clear fibrin network covering the entire extraction socket (Fig. 1a). Periodontal ligament was observed in the extraction socket together with residual endothelial cells around it. Neuronal NOS-negative fibroblasts were present in the extraction socket (Fig. 2a). These fibroblasts were positive for iNOS, eNOS, VEGF-C, or VEGFR-3 (Figs. 2b–e), and some were strongly positive for vWF (Fig. 2f).

In the Akita mouse group, fibroblasts had formed a clear fibrin network in the extraction socket, as noted in the control group. Periodontal ligament and blood vessels and residual endothelial cells around it were also observed in the extraction socket wall (Fig. 1d).

Fibroblasts in the extraction socket were negative for nNOS, showing no difference from those in the control group (Fig. 3a). The fibroblasts were only weakly positive for iNOS or VEGFR-3, showing a reduction in comparison with in the control group (Figs. 3b, e). The fibroblasts were also only negative or weakly positive for eNOS; many endothelial cells remaining in the periodontal ligament were also negative, while some cells were weakly positive (Fig. 3c); VEGF-C-positive fibroblasts and strongly vWF-positive fibroblasts were noted, showing no difference from those in the control group (Figs. 3d, f).

2) Day 4 after tooth extraction

The extraction socket was filled with granulation tissue, and many mesenchymal and endothelial cells were present, forming new blood vessels, including capillaries. New bone

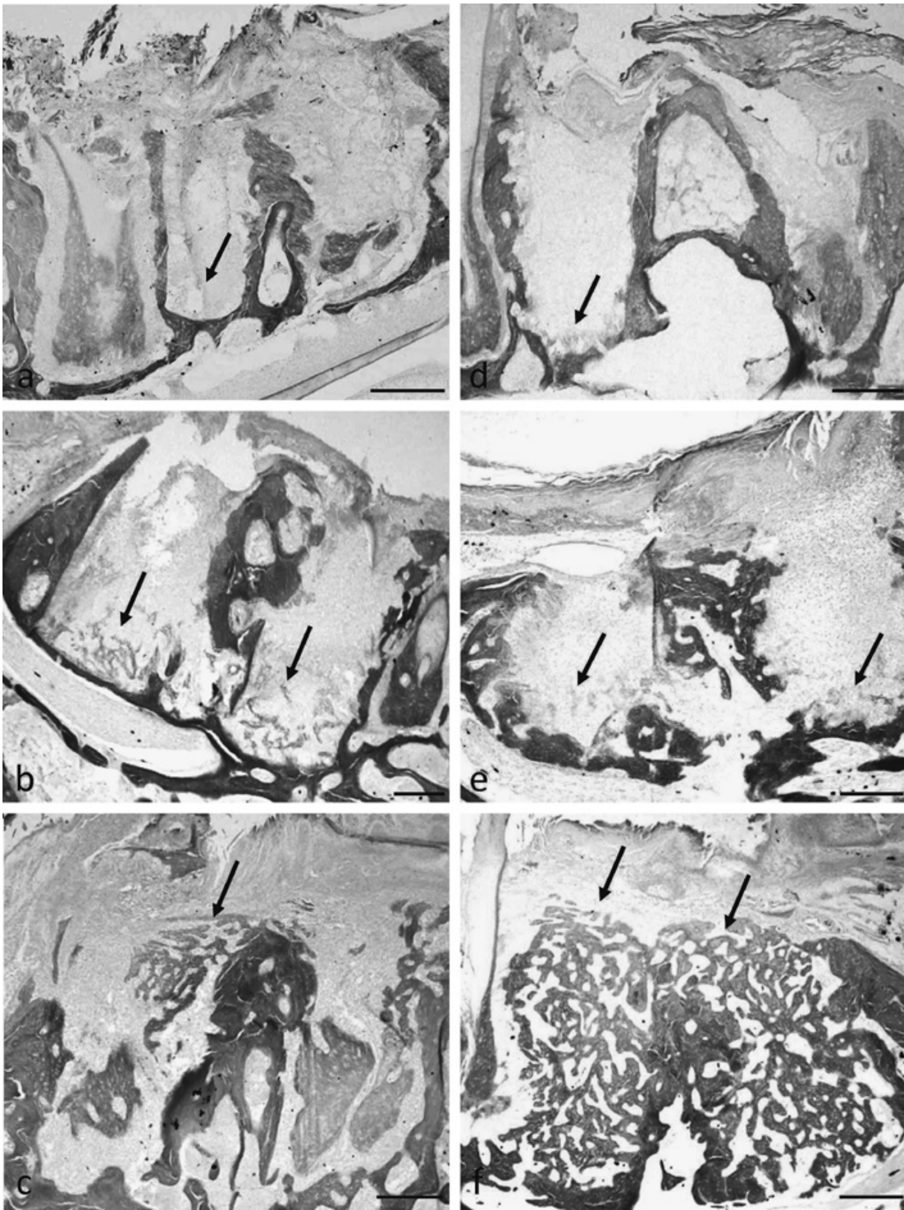

Fig. 1 Comparison of bone formation in extraction socket between control and Akita mouse groups (nNOS stain)

a: No bone formation was noted on day 1 after extraction in control group (black arrow), b: Small amount of new bone formed from root apex over central region on day 4 in control group (black arrow), c: New bone formation progressed toward central region on day 10 in control group (black arrow), d: No bone formation was noted on day 1 after extraction in Akita mouse group (black arrow), e: New bone formation was noted in root apex on day 4 in Akita mouse group (black arrow), f: Large amount of new bone (trabeculae) formed in reticular pattern over entire extraction socket on day 10 in Akita mouse group (black arrow).
Scale bar = 200 μm

formation was noted near the bottom of the socket, but it was sparse (Fig. 1b). Mesenchymal and endothelial cells showed a negative reaction for nNOS in the extraction socket,

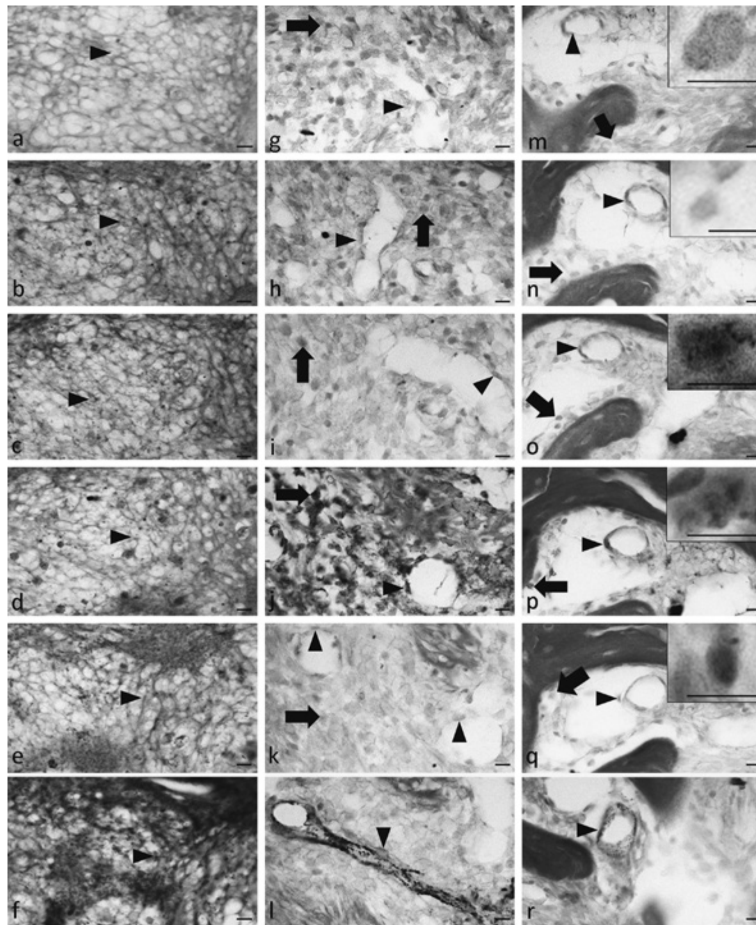

Fig. 2 Immunohistochemical staining of extraction socket tissue on days 1 (a–f), 4 (g–l), and 10 (m–r) after extraction in control group (magnified cells were osteoblasts)

a: nNOS-negative mesenchymal cells (black arrowhead) assumed to be fibroblasts were observed, b: iNOS-positive fibroblasts (black arrowhead) were observed, c: eNOS-positive fibroblasts (black arrowhead) were observed, d: VEGF-C-positive fibroblasts (black arrowhead) were observed, e: VEGFR-3-positive fibroblasts (black arrowhead) were observed, f: On staining with anti-vWF antibody, fibroblasts were positive (black arrowhead), g: Fibroblasts (black arrow) and endothelial cells (black arrowhead) were nNOS-negative, h: Fibroblasts (black arrow) and endothelial cells (black arrowhead) were weakly positive for iNOS, i: Fibroblasts (black arrow) and endothelial cells (black arrowhead) were weakly positive for eNOS, j: VEGF-C-positive fibroblasts (black arrow) and endothelial cells (black arrowhead) were observed, k: Fibroblasts (black arrow) and endothelial cells (black arrowhead) were weakly positive for VEGFR-3, l: Endothelial cells strongly positive for vWF (black arrowhead) were observed, m: Osteoblasts (black arrow) and endothelial cells (black arrowhead) were nNOS-negative, n: Osteoblasts (black arrow) were iNOS-negative. Endothelial cells weakly positive for iNOS (black arrowhead) were observed, o: Osteoblasts (black arrows) and endothelial cells (black arrowhead) weakly positive for eNOS were observed, p: Both osteoblasts (black arrow) and endothelial cells (black arrowhead) were VEGF-C-positive, q: Osteoblasts (black arrow) and endothelial cells (black arrowhead) weakly positive for VEGFR-3 were observed, r: Endothelial cells (black arrowhead) weakly positive for vWF were observed.
Scale bar = 10 μm

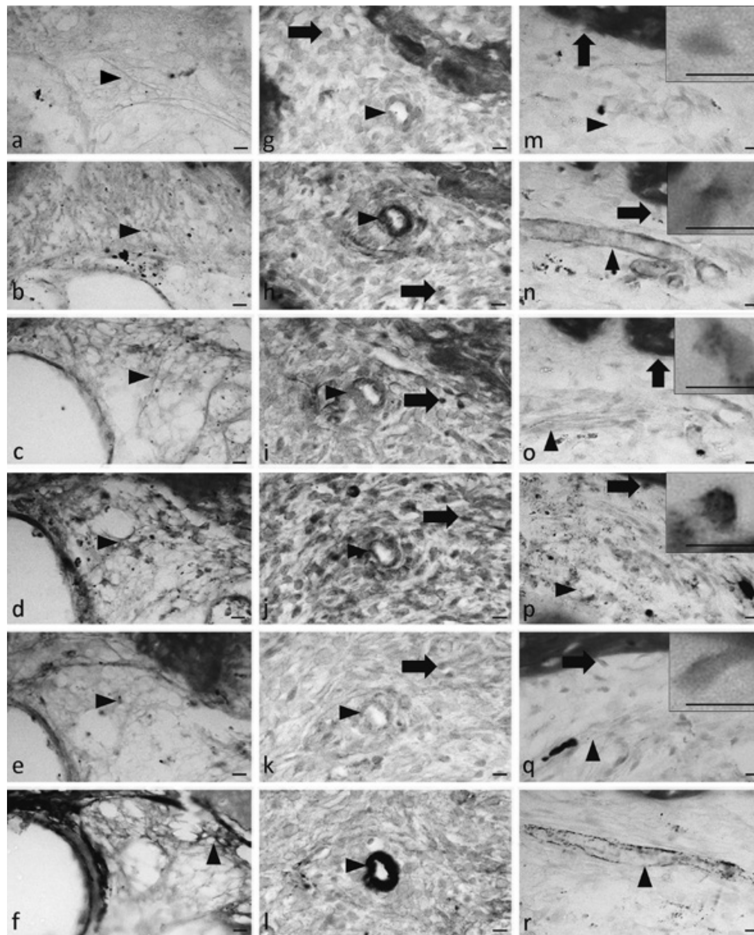

Fig. 3 Immunohistochemical staining of extraction socket tissue on days 1 (a–f), 4 (g–l), and 10 (m–r) after extraction in Akita mouse group (magnified cells were osteoblasts)

a: nNOS-negative mesenchymal cells (black arrowhead) assumed to be fibroblasts were observed, b: Fibroblasts (black arrowhead) weakly positive for iNOS were observed, c: eNOS-negative fibroblasts (black arrowhead) were observed, d: VEGF-C-positive fibroblasts (black arrowhead) were observed, e: Fibroblasts (black arrowhead) weakly positive for VEGFR-3 were observed, f: vWF-positive fibroblasts (black arrowhead) were observed, g: Fibroblasts (black arrow) and endothelial cells (black arrowhead) were nNOS-negative, h: iNOS-positive fibroblasts (black arrow) and endothelial cells (black arrowhead) were observed, i: eNOS-positive fibroblasts (black arrow) and endothelial cells (black arrowhead) were observed, j: VEGF-C-positive fibroblasts (black arrow) and endothelial cells (black arrowhead) were observed, k: Fibroblasts (black arrow) and endothelial cells (black arrowhead) weakly positive for VEGFR-3 were observed, l: Endothelial cells (black arrowhead) strongly positive for vWF were observed, m: Osteoblasts (black arrow) and endothelial cells (black arrowhead) were nNOS-negative, n: For iNOS, weakly positive osteoblasts (black arrow) and positive endothelial cells (black arrowhead) were observed, o: For eNOS, weakly positive osteoblasts (black arrow) and positive endothelial cells (black arrowhead) were observed, p: VEGF-C-positive osteoblasts (black arrow) and endothelial cells (black arrowhead) were observed, q: For VEGFR-3, weakly positive osteoblasts (black arrow) and endothelial cells (black arrowhead) were observed, r: vWF-positive endothelial cells (black arrowhead) were observed.
Scale bar = 10 μm

indicating no change from at day 1 (Fig. 2g). Some fibroblasts and endothelial cells were weakly positive for iNOS, eNOS, or VEGFR-3 (Figs. 2h, i, k). The number of fibroblasts and

endothelial cells positive for VEGF-C showed an increase (Fig. 2j). Vascular endothelial cells were strongly positive for vWF; iNOS-, eNOS-, VEGF-C-, and VEGFR-3-positive endothelial cells were strongly positive for vWF (Fig. 2l).

Similarly, the extraction socket was filled with granulation tissue in the Akita mouse group, and there were many fibroblasts and endothelial cells forming new blood vessels. New bone or osteoid formation near the extraction socket bottom was noted in some animals (Fig. 1e). Fibroblasts and endothelial cells in the extraction socket were negative for nNOS, showing no difference to those in the control group (Fig. 3g).

Some fibroblasts and endothelial cells were positive for iNOS, eNOS, or VEGF-C (Figs. 3 h–j). On the other hand, fibroblasts were weakly positive and endothelial cells positive for VEGFR-3 (Fig. 3k). Endothelial cells were strongly positive for vWF, and the reaction was slightly stronger than that in the control group (Fig. 3l).

3) Day 10 after tooth extraction

In the extraction socket in the control group, new bone formation progressed toward the central region (Fig. 1c). The trabeculae were surrounded by osteoblasts, connective tissue, and endothelial cells, and the lumen had expanded. The reaction with nNOS was negative in osteoblasts and endothelial cells, showing no difference from at day 4 (Fig. 2m). Many osteoblasts were negative for iNOS, while many endothelial cells were weakly positive or negative (Fig. 2n). Some osteoblasts and endothelial cells were weakly positive for eNOS or VEGFR-3 (Figs. 2o, q). Some osteoblasts and endothelial cells were positive for VEGF-C (Fig. 2p). While some endothelial cells were vWF-positive, many were only weakly so (Fig. 2r).

In the extraction socket in the Akita mouse group, new bone formation progressed strongly, with bone network covering the entire region and an increase in density (Fig.1f). The trabeculae were surrounded by osteoblasts, and many vascular endothelial cells were present in the bone marrow. Osteoblasts and endothelial cells were nNOS-negative, similarly to those in the control group (Fig. 3m). Osteoblasts were observed to be weakly positive and endothelial cells positive for iNOS or eNOS (Figs. 3n, o), showing stronger reactions than those in the control group. Osteoblasts and endothelial cells positive for VEGF-C were noted (Fig. 3p), showing no difference from those in the control group. Osteoblasts and endothelial cells were weakly positive for VEGFR-3 (Fig. 3q). The reaction with vWF was similar to those with eNOS, iNOS, or VEGF-C: vascular endothelial cells were positive (Fig. 3r), and the reaction was stronger than that in the control group.

2. Statistical analysis

Between days 1 to 10 after tooth extraction in the control group, although many cells were iNOS-positive at day 1, this decreased with time (Fig. 4a). Cells positive for eNOS showed the most marked decrease at day 4, but increased again by day 10 (Fig. 4b). Cells positive for VEGF-C showed an increase at days 4 and 10 compared with at day 1 (Fig. 4c). No significant change was noted in the number of VEGFR-3- or vWF-positive cells at any day (Figs. 4d, e).

In the Akita mice, the largest numbers of iNOS-, VEGF-C-, VEGFR-3-, and vWF-positive cells were noted at day 4, with positive cells decreasing at day 10 (Figs. 4a, c–e). Cells positive for eNOS showed an increase at day 4, with no change noted thereafter until day 10 (Fig. 4b).

Discussion

1. Expression of NOS during extraction socket healing process and appearance of lymphatic vessels

In the control mice, iNOS, eNOS, VEGF-C, VEGFR-3, and vWF were detected in fibroblasts and endothelial cells remaining in the periodontal ligament at day 1 after tooth extraction. Cells positive for iNOS, eNOS, or VEGFR-3 showed a significant decrease at day 4, but increased again at day 10. Cells positive

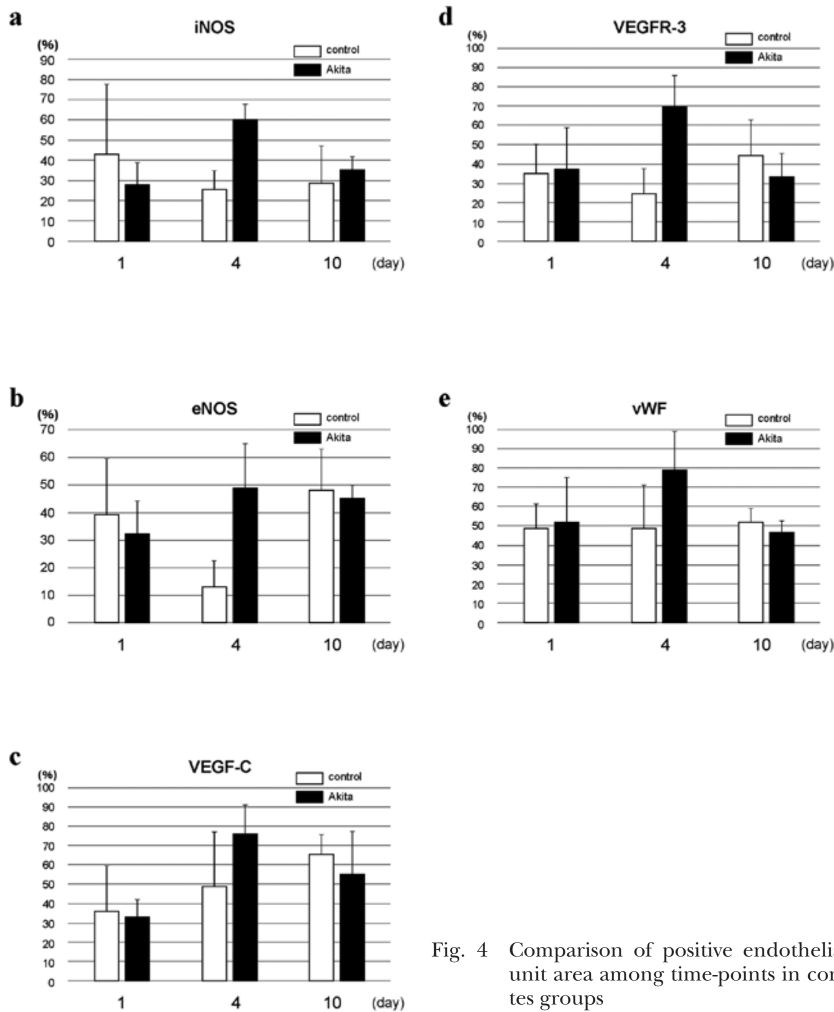

Fig. 4 Comparison of positive endothelial cell rate per unit area among time-points in control and diabetes groups

a: Comparison of iNOS-positive endothelial cell rate per unit area among time points in control and diabetes groups, Control: Positive endothelial cell rate was 43% on day 1 after extraction and between 25% and 30% on days 4 and 10, Diabetes: Positive endothelial cell rate was 27% on day 1 after extraction; it increased to 60% on day 4, but decreased to 35% on day 10, showing significant changes ($p<0.01$).

b: Comparison of eNOS-positive endothelial cell rate per unit area among time points in control and diabetes groups, Control: Positive endothelial cell rate was lower than 40% on day 1 after extraction; it decreased to 13% on day 4, but increased to 47% on day 10, Diabetes: Positive endothelial cell rate was 32% on day 1 after extraction; it increased to 48% on day 4, and slightly decreased to 44% on day 10.

c: Comparison of VEGF-C-positive endothelial cell rate per unit area among time points in control and diabetes groups, Control: Positive endothelial cell rate was 35% on day 1 after extraction; it increased to 48% on day 4 and 65% on day 10, Diabetes: Positive endothelial cell rate was 32% on day 1 after extraction; it increased to 77% on day 4, but decreased 55% on day 10.

d: Comparison of VEGFR-3-positive endothelial cell rate per unit area among time points in control and diabetes groups, Control: Positive endothelial cell rate was 35% on day 1 after extraction; it decreased to 24% on day 4, but again increased to 44% on day 10, Diabetes: Positive endothelial cell rate was 37% on day 1 after extraction; it increased to 70% on day 4, but decreased to 33% on day 10, showing significant changes ($p<0.05$).

e: Comparison of vWF-positive endothelial cell rate per unit area among time points in control and diabetes groups, Control: Positive endothelial cell rate was 49% on day 1 after extraction, 49% on day 4, and 52% on day 10, showing no marked change, Diabetes: Positive endothelial cell rate was 52% on day 1 after extraction; it increased to 79% on day 4, but decreased to 47% on day 10, showing significant changes ($p<0.05$).

for VEGF-C showed a time-dependent increase, whereas no significant change was noted in the number of vWF-positive cells. The development of inflammatory symptoms in the extraction socket has been reported to activate iNOS[16]. Moreover, another study has shown that an increase in eNOS, VEGF-C, and VEGFR-3 induces activation of the blood and lymphatic vasculature[1].

This suggests that the fibroblasts observed here were involved in angiogenesis and lymphangiogenesis, inducing precursors of vascular and lymphatic endothelial cells. Expression levels of LYVE-1, podoplanin, Prospero homeobox protein 1 (Prox-1), and VEGFR-3 showed a marked increase during the development of lymphatic vessels[13], while iNOS and eNOS were expressed in lymphatic endothelial cells[20]. In addition, reduced expression of NOS decreased lymphatic flow stimulation[9]. Thus, NOS is closely associated with lymphatic vessels.

In the present study, the number of VEGFR-3-positive cells grew with increase in iNOS and eNOS at day 10 after tooth extraction, suggesting active regeneration of lymphatic vessels. An increase was also observed in the number of VEGF-C-positive endothelial cells, which was consistent with the findings of an earlier study showing that inflammation-inducing cytokines promoted VEGF-C production[17,24]. In the present study, the number of vWF-positive cells remained constant throughout, suggesting that formation of many blood vessels is necessary in the healing process after tooth extraction, and that lymphatic vessels are formed as blood vessels increase.

In the Akita mice, the largest numbers of endothelial and undifferentiated mesenchymal cells positive for markers other than nNOS were present at day 4 after tooth extraction. Inflammatory reactions and NO actions may have been delayed at day 1 after tooth extraction compared with those in the control group, suggesting that cell differentiation and induction are delayed in diabetes. However, both iNOS and eNOS started to show an increase at day 4, suggesting close involvement in the development of inflammatory symptoms and the formation of the lumen by lymphatic endothelial cells.

Positivity to VEGF-C and VEGFR-3 also grew stronger. This suggested active induction of undifferentiated mesenchymal cells, which would later differentiate into lymphatic endothelial cells, and growth regulatory factor, promoting lymphangiogenesis. The results for vWF suggested its involvement in the differentiation and induction of vascular endothelial cells. Endothelial cells positive for vWF but negative for VEGF-C or VEGFR-3 were judged to be vascular endothelial cells, and many such cells were observed from day 1 to 10 in both the control and Akita mouse groups. Endothelial cells positive for VEGF-C or VEGFR-3 but negative for vWF were judged to be lymphatic endothelial cells. Very few cells, however, were assumed to be lymphatic endothelial cells during the blood clot, granulation tissue, or callus phases. On the other hand, endothelial cells positive for all three of VEGF-C, VEGFR-3, and vWF were observed.

As previously reported, lymphatic vessels sprout from veins[17], and VEGFR-2, a marker of vascular endothelial cells, and VEGFR-3, a marker of lymphatic endothelial cells, are expressed in early vascular endothelial cells. Vascular endothelial cells positive for VEGFR-3 have the potential to differentiate into lymphatic endothelial cells, but Prox-1 expression is necessary to induce actual differentiation. In regions expressing Prox-1, VEGFR-2 expression decreases, VEGFR-3 and podoplanin expression increase, and cells gather in VEGF-C-expressing regions and form lymphatic vessels[10,17]. The partial involvement of VEGF-C in angiogenesis in the fetal period has been reported[24]. Therefore, endothelial cells positive for all 3 markers, VEGF-C, VEGFR-3, and vWF, may have been present during differentiation from vascular to lymphatic endothelial cells.

Mesenchymal and endothelial cells were nNOS-negative at 1, 4, and 10 days after tooth extraction in the control and diabetes groups. Although some studies have suggested that nNOS is expressed in nerve fibers, and that

it protects blood vessels instead of iNOS in the vascular system[18,19,27], nNOS was negative in our study, suggesting that no such action occurred in the healing process following tooth extraction.

2. New bone formation

In contrast to in many earlier studies reporting that healing of extraction sockets is delayed in mouse diabetes models, bone formation here was marked. New bone and osteoid formation was observed at day 4 after tooth extraction in both the control and Akita mouse groups. Bone quality became markedly dense at day 10 in the Akita mouse group. Since VEGF-C positivity and weak VEGFR-3 positivity were detected in both the control and Akita mouse groups, proliferation and differentiation of osteoblasts may have been promoted. However, the low metabolic turnover that accompanies insulin deficiency has been reported to induce osteopenia due to disruption of osteoblastic and osteoclastic function[12].

It has also been reported that hyperglycemic conditions strongly enhance bone morphogenetic protein (BMP) activity, increasing bone-forming ability[4], and that BMP receptor binding is not inhibited or affected by insulin levels under hyperglycemic conditions[12]. Insulin deficiency disrupts osteoblastic and osteoclastic function. However, the present results suggest that promotion of osteoblastic differentiation and proliferation by BMP compensates for insulin deficiency-induced loss of such function, indicating that this would promote bone formation in extraction sockets in patients with diabetes.

However, glycation during bone formation promotes crosslinking of advanced glycation end products under hyperglycemic conditions[22], suggesting that bone formation is disrupted, increasing susceptibility to fracture and diseases such as osteoporosis and making bone strength insufficient to sustain implant treatment after tooth extraction. Thus, healing may not necessarily promote bone formation in a favorable direction. The present results showed that angiogenesis preceded lymphangiogenesis in both the control and Akita mouse groups.

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Conflict of Interest

The authors declare no conflict of interest.

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