ORIGINAL ARTICLE

Effects of Novel Synthetic Peptide during Early Wound Healing of Periodontal Artificial Defects in Rats

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SYNOPSIS
To clarify the function of novel synthetic peptide with an amino acid sequence the same as a partial sequence of amelogenin gene exon 5, we applied them to periodontal tissue defects artificially prepared in the rat molar region. Sections of the applied regions were prepared 3, 5, 7, and 14 days after surgery and histopathologically investigated. In addition, type III collagen and von Willebrand factor were immunohistochemically observed. Epithelial cell growth was promoted for 3 days after surgery and then inhibited thereafter. Fibroblast growth was advanced, type III collagen decreased in the early phase, and the ratio of blood vessels significantly increased, showing the promotion of vascularization. The novel synthetic peptide seems to promote the wound-healing process, inducing periodontal tissue regeneration in the early phase.

Key words: *novel synthetic peptide, early wound healing, type III collagen, von Willebrand factor*

INTRODUCTION
Emdogain® (EMD) is a freeze-dried protein purified from the tooth germs of 6-month-old pigs [1]. It is widely used clinically in periodontal tissue regeneration therapy, and favorable outcomes, such as improvement of the probing depth, attachment level, and intraosseous defects on radiography, have been reported [2,3]. Many *in vivo* and *in vitro* studies have clarified the usefulness of EMD for periodontal tissue regeneration, and the mechanism of such regeneration using EMD has been clarified [4,5]. However, it is possible that unknown substances are also present in the product, and some patients reject it because it is derived from animal. Moreover, EMD exhibits antigenicity due to mixed protein components, and treated patients produce anti-EMD antibodies [6].

Kim *et al.* [7] subcutaneously injected old-type EMD into the rat dorsal region, observed cartilage formation in the injected region after 7 days, and discovered eosinophilic round bodies (ERBs)

around the region. On analysis using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), ERBs were comprised of peptide fragments with various lengths of 7-192 residues, all peptide fragments contained a 7-amino acid sequence: WYQNMIR, and this was a partial sequence of amelogenin gene exon 5 [7]. When Hida *et al.* [8] synthesized a peptide comprised of these 7 amino acid residues (novel synthetic peptide) and subcutaneously injected it into the rat dorsal region, bone, cartilage, and enchondral ossification were noted after 2 weeks. This novel synthetic peptide was comprised of only the 7 residues, shorter than 10 residues, unlike EMD containing various proteins, and the molecular weight was 1,118 Da, lower than 5,000 Da. Therefore, there is no risk of antibody production [8-10]. *In vitro* studies [11-14] using the novel synthetic peptide clarified the action mechanisms on gingival epithelial cells, periodontal ligament fibroblasts, and vascular endothelial cells, but *in vivo* study has been scarcely reported, leaving many points to be clarified. In the present study, we investigated the influence of the novel synthetic peptide on the early phase of the wound-healing process of rat artificial periodontal tissue defects *in vivo*.

MATERIALS AND METHODS
1. Animals
Male Sprague-Dawley rats at 8 weeks after birth were purchased (Shimizu Laboratory Supplies Co., Kyoto, Japan) and acclimated with pellets and tap water for one week in an animal experimental facility of Osaka Dental University Institute of Dental Research, and 40 rats weighing about 250 g (20 each in experimental and control groups) were used in the experiment.

2. Materials
The novel synthetic peptide was prepared at a concentration of 15 mg/mL in 1.5 % propylene glycol alginate (PGA, Wako pure Chemical Industries Ltd., Osaka, Japan) solution [15] and used in the experiment. To the control group, 1.5% (w/v) PGA aqueous solution not containing the peptide was applied.

3. Methods
To a rat under inhalation anesthesia with isoflurane (Forane®, Abbott, North Chicago, U.S.A.), pentobarbital (Somnopentyl Injection®, Kyoritsu Seiyaku Co., Tokyo, Japan) was intraperitoneally injected at 0.3 mg/kg, and the rat was restrained with the mouth open in a supine position. Setting the target of observation as periodontal tissue including the palatal-side central roots of the bilateral upper first molars, surgery was performed as follows: An internal bevel incision was made in the central region of the mesial surface of the upper first molar toward the palatal side along the gingival sulcus over the palatal mesial angle of the second molar using a Slit Knife (Alcon, Hünenberg, Switzerland), and an about 1-mm transverse incision was extended in the proximal direction. A full-thickness flap comprised of mucoepithelium, lamina propria mucosae, and periosteum in this region was prepared using a dental excavator (Hu-Friedy, Chicago, U.S.A.). The alveolar bone, periodontal ligament, and root surface (cementum and dentin) were then removed from the palatal mesial angle of the first molar over the palatal mesial angle of the second molar using a round bar with a 1-mm diameter (ISO #010, Maillefer Co., Tokyo, Japan) under irrigation. The residual root surface was scraped with a double-headed chisel (Ochsenbein #3, Hu-Friedy) and sufficiently washed with saline. One pre-suture was incompletely applied to the mesial first molar using 6-0 absorb-

able thread (VICRYL[®], Ethicon Inc., New Jersey, U.S.A.) [16,17]. PGA containing the novel synthetic peptide and PGA alone were applied to the defects in the experimental and control groups, respectively, the gingival full-thickness flap was returned to the original position, and the pre-suture was completely sewn. After that the surgical region was pressed with a swab for about 3 minutes for hemostasis. Five animals each were euthanized 3, 5, 7, and 14 days after surgery by intraperitoneal pentobarbital overdose under isoflurane inhalation anesthesia in each of the experimental and control groups. This study was performed in conformity with the Guidelines for Proper Conduct of Animal Experiments and Related Activities after approval by the Animal Research Committee of Osaka Dental University (approval number: 14-01002).

4. Tissue preparation

After being euthanized, thoracotomy was immediately performed, a catheter was inserted into the ascending aorta through the left ventricle, and 10% neutral buffered formalin (Nacalai Tesque Inc., Kyoto, Japan) was perfused for fixation. The test root and surrounding tissue were excised en bloc, and immersed in fresh fixative at 4℃ for 7 days. The sample was then decalcified using K-CX (Falma Co., Ltd., Tokyo, Japan) for 24 hours at 4℃. After decalcification, unnecessary tissue was removed, and the sample was divided in the buccopalatal direction on the mesial side of the observation site: the palatal central root of the first molar. The divided samples were washed with 0.1 M phosphate buffer (PBS) adjusted to pH 7.2 at 4℃ and then paraffin-embedded following the routine method. Serial sections with a 4-μm thickness in the longitudinal direction were prepared to include the apex of the palatal central root of the first molar.

5. Staining of sections

Regarding 3 adjacent thin sections as one set, 3 sets were prepared in both the experimental and control groups, and each set was subjected to hematoxylin-eosin (H-E) staining and immunohistochemical staining with antibodies against type III collagen and von Willebrand factor (factor VIII). In immunostaining, after deparaffinization, sections were reacted with 3% H_2O_2 for 15 minutes at room temperature to inactivate endogenous peroxidase. To activate antigens, sections for immunostaining of type III collagen were reacted with pepsin adjusted to 0.04% with 0.01 N HCl for 60 minutes at 37℃, and those for immunostaining of von Willebrand factor were reacted with proteinase K (Dako, Glostrup, Denmark) for 5 minutes at room temperature. The sections were then reacted with rabbit anti-type III collagen polyclonal antibody (Novus BIOLOGICALS, Colorado, U.S.A.) diluted 100 times with Dako REAL Antibody Diluent (Dako, Glostrup, Denmark) [18] for 60 minutes at 37℃ and rabbit anti-von Willebrand polyclonal antibody (Dako, Glostrup) diluted 1,000 times with the same diluent for 60 minutes at room temperature, respectively, followed by reaction with En Vision Labelled Polymer Peroxidase (Dako, Glostrup) for 30 minutes at 37℃ and 60 minutes at room temperature, respectively, and color development with 3,3,-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Ltd., Kumamoto, Japan). The sections were then stained with hematoxylin for nuclear staining, dehydrated, mounted, and observed under a light microscope.

6. Comparison of the number of blood vessels

The numbers of blood vessels were arbitrarily quantified to compare them between the experimental and control groups.
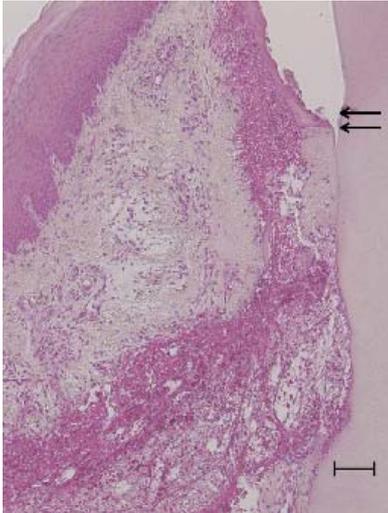
Fig. 1 Control group at 3 days after surgery, Hematoxylin and eosin (HE) stain, Original magnification ×10, Bar= 100μm

The wound surface is not covered by gingival sulcus epithelium, and no gingival junctional epithelium had regenerates. (arrows)

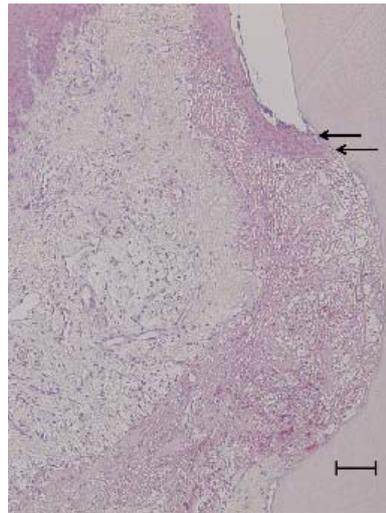**Fig. 2** An experimental group at 3 days after surgery, Hematoxylin and eosin (HE) stain, Original magnification ×10

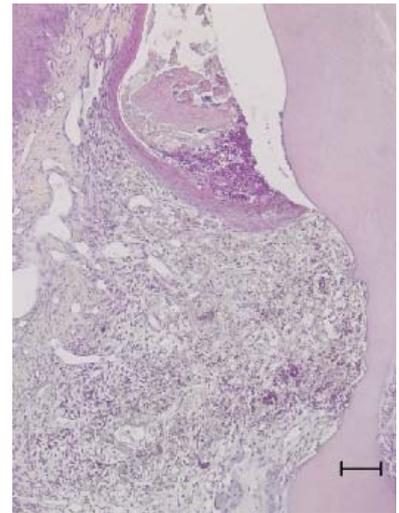Both the gingival sulcus and junctional epithelia regenerates and covered the wound surface. (arrows)

Fig. 3 Control group at 5 days after surgery, Hematoxylin and eosin (HE) stain, Original magnification ×10

The wound surface is covered by gingival sulcus epithelium, and gingival junctional epithelium regenerates.

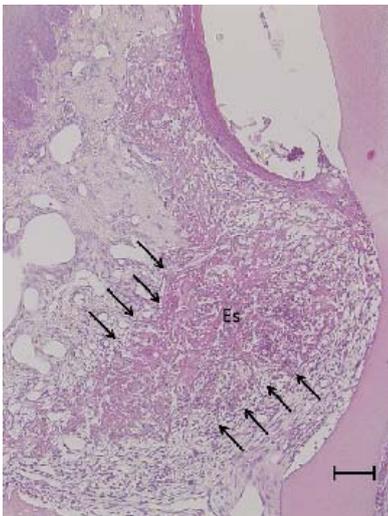**Fig. 4** An experimental group at 5 days after surgery, Hematoxylin and eosin (HE) stain, Original magnification ×10

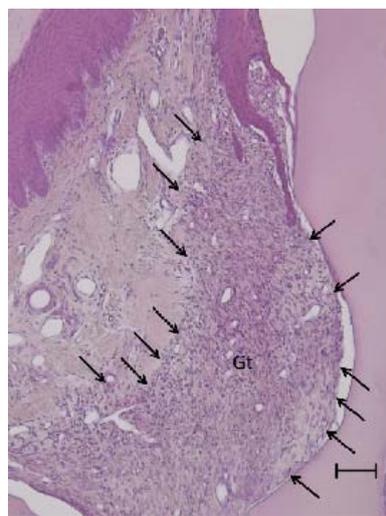An eosinophilic substance (Es) accumulates in some regions. (arrows)

Fig. 5 Control group at 7 days after surgery, Hematoxylin and eosin (HE) stain, Original magnification ×10

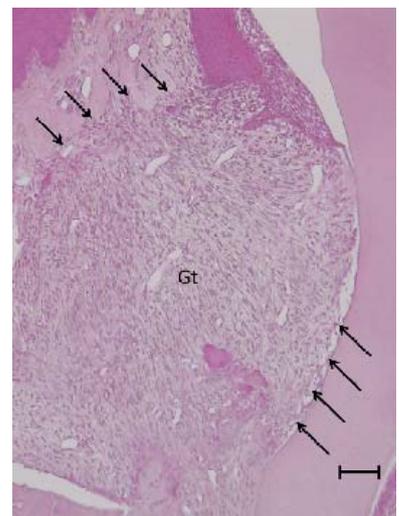Granulation tissue (Gt) (arrows) containing many fibroblasts but only a few collagen fibers are formed.

Fig. 6 An experimental group at 7 days after surgery, Hematoxylin and eosin (HE) stain, Original magnification ×10

Fiber components are abundant, and collagen fibers distributed almost vertical to dentin are partially observed. (arrows)
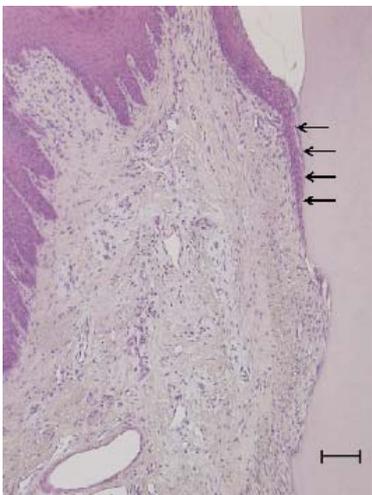

Fig. 7 Control group at 14 days after surgery, Hematoxylin and eosin (HE) stain, Original magnification ×10

Downward growth of gingival junctional epithelium is noted. (arrows)

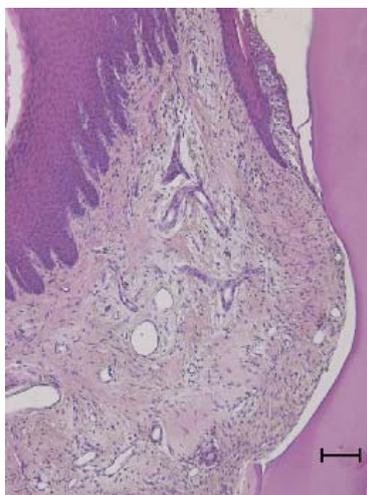**Fig. 8** An experimental group at 14 days after surgery, Hematoxylin and eosin (HE) stain, Original magnification ×10

No downward growth of gingival junctional epithelium is discernible.

Microscopic images of 3 sections immunostained with anti-von Willebrand factor antibody of each test region were input using a digital image input device (3CCD 2Mega Pixel Digital Camera FX630, Olympus Optical Co., Ltd., Tokyo, Japan), and the total number of vessels and number of blood vessels per unit area were counted.

Lumen-like structures containing flowing extracellular fluid, such as blood and lymph, were regarded as vessels, and all arteries, veins, and capillary blood vessels with vascular endothelial cells positive for von Willebrand factor on immunostaining were regarded as blood vessels.

The number of blood vessels as a percentage of the total number of vessels was calculated as follows:

(Number of von Willebrand factor-
  positive vessels (blood vessels) /
  total number of vessels) x 100 (%)

For the observation range, at 10-times magnification of the object lens, the left side was set at the most lateral site of the defect and the lower side was set at the basal region of the defect. The results of calculation were compared between the experimental and control groups using Student's t-test.

RESULTS
1. Histopathological findings
1) Day 3 after surgery
In the control group, blood clots were noted in the injured periodontal tissue region surrounded by the excised root surface, periodontal ligament, alveolar bone, and inner wall of the gingival flap, and fibrin containing many neutrophils had formed a network structure. Gingival sulcus epithelium had not closed the wound surface, and no gingival junctional epithelium had regenerated (Fig. 1).

In the experimental group, blood clots contained fewer inflammatory cells compared to those in the control group. Both the gingival sulcus and junctional epithelia had regenerated and covered the wound surface (Fig. 2).

2) Day 5 after surgery
In the control group, granulation tissue containing abundant vessels and inflammatory cells was noted in the periodontal tissue defects. Gingival sulcus epithelium covered the wound surface, and the gingival junctional epithelium had regenerated (Fig. 3).

In the experimental group, an eosinophilic substance accumulated in a part of the periodontal tissue defect as granulation tissue was formed (Fig. 4).

3) Day 7 after surgery
In the control group, the periodontal tissue defects were filled with the granulation tissue containing more cell components than in granulation tissue on day 5. The granulation tissue contained many cell components, particularly fibroblasts, but only slight collagen fiber formation was noted (Fig. 5).

In the experimental group, the width of the gingival junctional epithelium covering the wound surface was narrower than that in the control group, and it was similar to that on day 5. The granulation tissue in the periodontal tissue defects contained abundant fiber components compared to that in the control group, and collagen fibers mostly vertical to the dentin were partially observed (Fig. 6).

4) Day 14 after surgery
In the control group, the gingival junctional epithelium grew downward compared to that in the experimental group (Fig. 7), but it was not noted in the experimental group (Fig. 8). No marked difference was noted in granulation tissue in the periodontal tissue defects between the control and experimental groups.
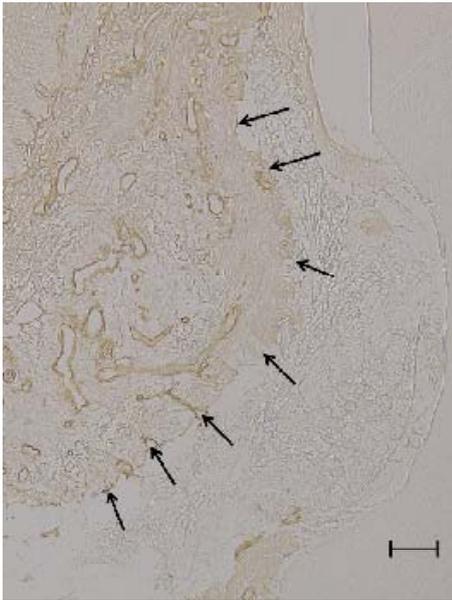
Fig. 9 An experimental group at 3 days after surgery, Anti-type Ⅲ collagen Ab stain, Original magnification ×10

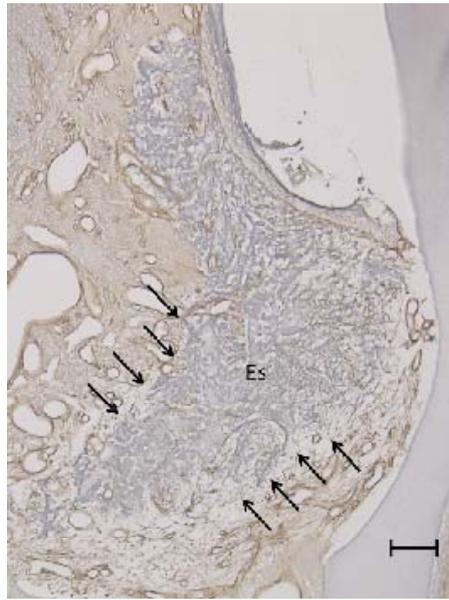Type III collagen-positive reaction is noted in existing connective tissue in contact with blood clots. (arrows)

Fig. 10 An experimental group at 5 days after surgery, Anti-type Ⅲ collagen Ab stain, Original magnification ×10

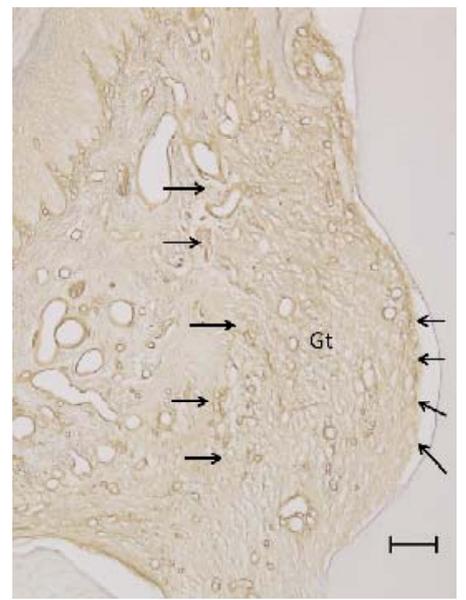The eosinophilic substance observed in Fig. 4 is type III collagen-negative. (arrows)

Fig. 11 Control group at 7 days after surgery, Anti-type Ⅲ collagen Ab stain, Original magnification ×10

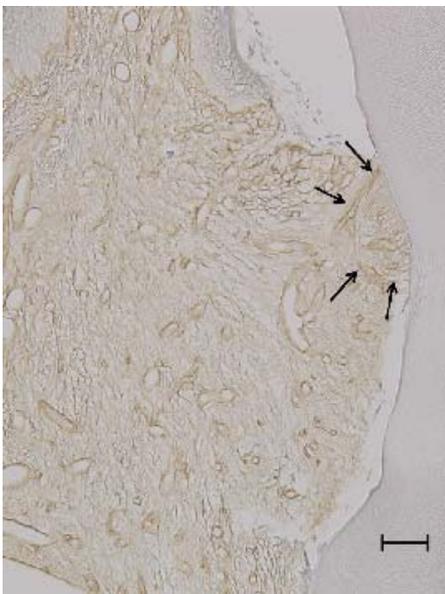Type III collagen-positive reaction is observed in granulation tissue. (arrows)

Fig. 12 An experimental group at 7 days after surgery, Anti-type Ⅲ collagen Ab stain, Original magnification ×10

Fibers distributed almost vertical to dentin are type III collagen-positive. (arrows)
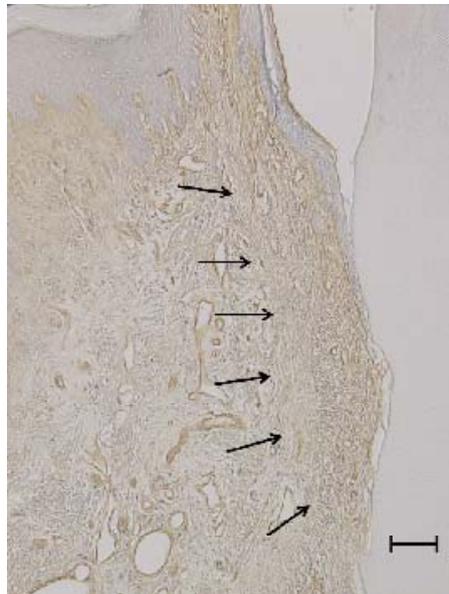
Fig. 13 Control group at 14 days after surgery, Anti-type Ⅲ collagen Ab stain, Original magnification ×10

Type III collagen-positive reaction at a level similar to that in Fig. 11 is discernible. Type III collagen-positive area (arrows)

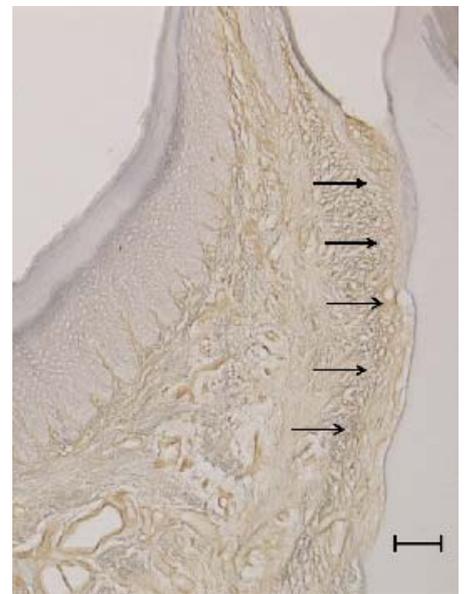**Fig. 14** An experimental group at 14 days after surgery, Anti-type Ⅲ collagen Ab stain, Original magnification ×10

Type III collagen-localized regions decrease compared to those in Fig. 13. Type III collagen-positive area (arrows)

2. Findings of immunohistochemical staining with anti-type III collagen antibody

1) Day 3 after surgery

No difference was noted between the control and experimental groups. In both groups, type III collagen was localized in the existing connective tissue in contact with blood clots in the defects (Fig. 9).

2) Day 5 after surgery

Type III collagen was localized in the existing connective tissue and granulation tissue formed in the periodontal tissue defects in the control and experimental groups.

The eosinophilic substance observed in the experimental group on H-E staining was type III collagen (Fig. 10).

3) Day 7 after surgery

Type III collagen was localized in the existing connective tissue and granulation tissue formed in the periodontal tissue defects in the control and experimental (Fig. 11) groups.

In the experimental group, fibers distributing almost vertical to the dentin surface in the periodontal tissue defects were positive for type III collagen (Fig. 12).

4) Day 14 after surgery

In the control group, the type III collagen-positivity level was similar to that on day 7 in gingival connective tissue directly below the epithelium (Fig. 13). In the granulation tissue formed in the periodontal tissue defects, the type III collagen-localized regions decreased in the experimental group compared to those in the control group (Fig. 14).

3. Rate of blood vessels and statistical analysis

The number of blood vessels expressed as a percentage of the total number of vessels in the periodontal tissue defects on day 3 after surgery was $75.8\pm7.4\%$ in the control group and $77.5\pm6.7\%$ in the experimental group, showing no significant differences between them. The rates were 68.9 ± 5.7 and $80.5\pm2.7\%$ on day 5, respectively ($p<0.017$), 61.9 ± 11.9 and $81.1\pm1.6\%$ on day 7, respectively ($p<0.025$), and 53.8 ± 13.1 and $81.0\pm10.4\%$ on day 14, respectively ($p<0.024$), showing significant differences at all time-points (Fig. 15).
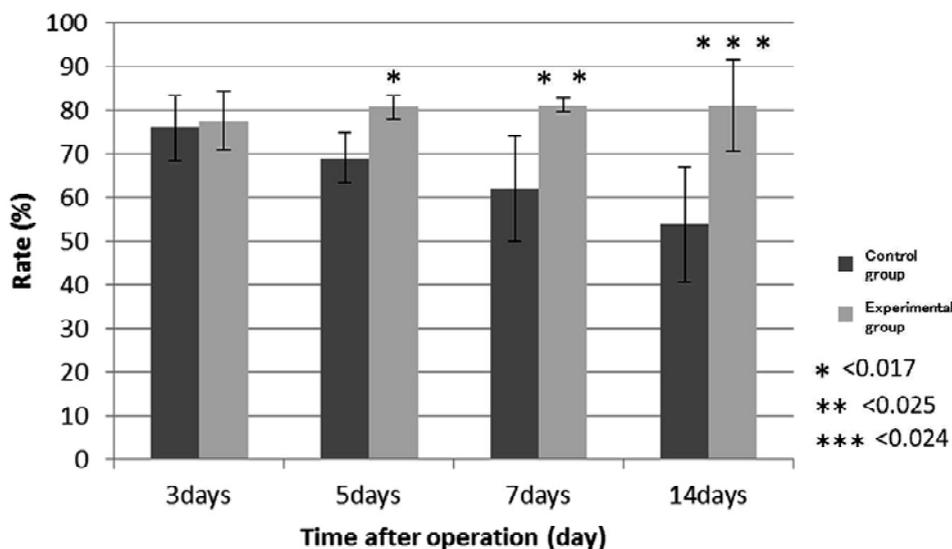

Fig. 15 Comparison of the number of blood vessels.

DISCUSSION

In wound healing of periodontal tissue, the regeneration state is determined by 4 types of cell: epithelial, gingival connective tissue-derived, bone-derived, and periodontal ligament-derived cells [19].

Wounds healed rapidly in the experimental group because the wound surface was covered by epithelium within 3 days after surgery, and the wounds were filled with blood clots containing fewer inflammatory cells. On day 5 after surgery, many fibroblasts were present compared to those in the control group, and the tendency towards fibrosis was noted. Fibrosis had progressed on day 7, promoting healing. Type III collagen was localized in granulation tissue formed in both control and experimental groups, and it was observed from day 5 to 7. Type III collagen localization was decreased on day 14 in the experimental group. There were more blood vessels in the experimental than in the control group.

Generally, when the wound site is not covered by epithelial tissue, connective tissue in the regenerated region is exposed to the oral cavity, which causes persistent infection and delays healing [20], i.e., rapid closure of wounds by epithelial tissue promotes the healing process. On day 3 after surgery, gingival sulcus epithelium had not covered the wound surface in the control group, but it had covered the wound surface in the experimental group. The novel synthetic peptide promoted the growth of epithelial cells for 3 days until the wound surface was covered, suggesting that it promotes wound healing. Actually, epithelial cell proliferation is more active than that of fibroblasts in the early wound-healing process, showing that the cells rapidly react to injury [19].

However, epithelial cell growth promotes an epithelial shift toward the root apex (downward growth), forming the long attachment of junctional epithelium. Therefore, to achieve connective tissue attachment in the wound-healing process, it is necessary to inhibit epithelial growth [21,22]. When human gingival epithelial cells were cultured with the novel synthetic peptide, epithelial cell growth was inhibited [13]. The existence of long junctional epithelium due to its downward growth was noted on days 7 and 14 after surgery in the control group, but no downward epithelial growth was noted in the experimental group, and this was consistent with the action of the novel synthetic peptide observed *in vitro*, i.e., the epithelial cell growth-inhibitory effect [13]. The novel synthetic peptide may have inhibited epithelial cell growth after day 7, preventing the formation of long junctional epithelium.

Wound closure in the early wound-healing process involves epithelial cell growth, but it is considered that type III collagen is not involved in this [23]. In our experiment, wound closure by epithelium was noted on day 3 only in the experimental group, but no change was noted in the stainability of type III collagen in either the experimental or control group, showing that type III collagen involvement in wound closure by epithelial cells is unlikely.

Type III collagen localization in the periodontal tissue defects had decreased on day 14 in the experimental group compared to that in the control group. Type III collagen is initially produced in the wound and then disappears through replacement by tough type I collagen [24]. Type III collagen disappeared earlier than in the control group, suggesting that the novel synthetic peptide advanced the wound-healing process.

When human periodontal ligament fibroblasts were cultured with the novel synthetic peptide, the DNA level increased [11], and this is consistent with our finding that granulation tissue com-

prised of abundant fibroblasts was formed on days 5 and 7 in the experimental group compared to that in the control group.

Cell growth factors involved in periodontal tissue regeneration include platelet-derived growth factor (PDGF) [25-27], transforming growth factor-*β* (TGF-*β*) [27], fibroblast growth factor (FGF) [28-30], epidermal growth factor (EGF) [31,32], and insulin-like growth factor (IGF) [33-35]. These growth factors are produced by periodontal ligament cells and promote or inhibit cell growth, differentiation, angiogenesis, and the function of the extracellular matrix [36]. When periodontal ligament cells grow in response to the novel synthetic peptide [11,12], the secretion of these growth factors may also increase [36].

Since injured tissue recovers from a hypoxic condition as blood flow resumes with the progression of wound healing, with which the expression of VEGF induced by the reduced tissue oxygen level decreases [37]. The number of blood vessels as a percentage of the total number of vessels was significantly higher in the experimental than in the control group on days 5 to 14, and this was consistent with the finding of the *in vitro* experiment: human microvasculature endothelial cells showed significantly increased growth when cultured with the novel synthetic peptide [14].

Normally, when blood flow resumes in wounds, excess blood vessels disappear because they are not covered by pericytes. Actually, the rate of blood vessels decreased with time in the control group. In contrast, the rate did not decrease in the experimental group. Although it was not confirmed in our study, hard tissue was formed 14 days after application of the novel synthetic peptide *in vivo* [8.15]. For vascular endothelial cells, the peptide promoted cells to grow and increase the number of blood vessels, which maintained blood supply to the defective regions and provided abundant oxygen and nutrients to form hard tissue, i.e., it was suggested that the novel synthetic peptide promotes periodontal tissue regeneration through acting on the early wound-healing process.

It was clarified that the novel synthetic peptide advanced coverage of the wound surface by epithelial cells while inhibiting downward epithelial growth. For fibroblasts, the peptide promoted cells to grow and secrete growth factors to form connective tissue attachment.

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