Periodontal regeneration in intrabony defects after application of enamel matrix proteins with guided tissue regeneration: An experimental study in dogs

Hiroko ONODERA, Yoshihiro SHIBUKAWA, Hiroki SUGITO, Mikio OTA and Satoru YAMADA
Department of Periodontics, Tokyo Dental College, Chiba, Japan

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ABSTRACT
The aim of this study was to evaluate the effects of enamel matrix proteins (EMP) at the early stage of wound healing in the periodontal tissues by a combination treatment with guided tissue regeneration (GTR). Intrabony defects were produced surgically at the distal aspects of both mandibles in six beagle dogs. At 12 weeks following the surgery, the defects were exposed using a full thickness mucoperiosteal flap procedure. Subsequently, the defects were treated by the following treatments: a control group treated with GTR alone, and an experimental group treated with a combination of GTR and EMP. After one, two, four and eight weeks of the treatment, the animals were sacrificed, and sections of the tissue were stained and evaluated microscopically. After one and two weeks, the proliferating cell nuclear antigen (PCNA)-positive cell ratios of the experimental group were significantly greater than that of the control group. After 2 and 4 weeks, new bone and new cementum formation in the experimental group were significantly greater than those in the control group (P < 0.05). However, after 8 weeks, no statistical difference was found between the two groups in new bone or cementum formation. The study results suggest that a maturation of periodontal ligament cells might contribute, during the early stage of periodontal healing, to stimulate a proliferation of periodontal ligament cells.

Periodontal regeneration therapy aims at the recovery of periodontal tissues lost due to inflammatory periodontal disease (4). This type of healing requires the formation of new connective tissue (for example, newly generated cementum and alveolar bone attached to collagen fibrils) (4, 14). Based on the recent studies, it is believed that the most useful therapy that increases regeneration of periodontal tissues is guided tissue regeneration (GTR) (14, 15). In GTR, a protective membrane is placed between the exposed root surface and the periodontal tissue to provide a selective isolated space, which accelerates repopulation of cells from the periodontal ligament and alveolar bone cells in that space (15, 16). GTR therapy for bone defects forms new cementum that is attached to connective tissues in many situations. Histological and clinical studies have shown that 2 or 3-wall bone defects and class-II lesions at the furcation in the mandibles are regenerated with appropriate predictions (3, 6, 7, 11, 13). However, the outcome seems to be poor when GTR was applied to 1-wall bone defects or to maxillary class-II or mandibular class-III lesions at the furcation (2, 18, 21).

Recent studies have demonstrated that enamel matrix protein (EMP) induces the formation of cellular cementum in defects surrounded by dentin and alveolar bone in monkeys and in the minipigs (8, 9). Proliferation, protein production and calcification of periodontal ligament cells have been shown in vitro,
which supports the hypothesis on clinical usefulness of EMP. The safety and effectiveness of EMP for improving the repair of periodontal bone defects have been noted in humans and other animals (17, 23), and a histological study reported that the true periodontal regeneration ability was maintained (12). The adjuvant use of EMP in regeneration therapy aimed at the formation of cementum, alveolar bone and periodontal membrane structure is expected. Araújo and Lindhe (1) reported that a combination of EMP and GTR induced the regeneration of class-III lesions at the furcation. From the clinical aspects, the composition of EMP with GTR is very interesting with regard to its regeneration ability. It has been demonstrated that EMP affects the proliferation and maturation of periodontal ligament cells at the early stage of wound healing (19). Gestrelius et al. (10) has also indicated that the repopulation of periodontal ligament cells is promoted at the first week after EMP application. These previous reports have suggested that EMP acts significant role at the early stage of wound healing in the periodontal ligament cells. On the other hand, a contradict result has been displayed that the effect of EMP appears in the periodontal tissue 4 months after the treatment with EMP and GTR (1). Thus, it is an open question whether EMP exhibits the effect at the early stage of wound healing in the periodontal tissues under an application with EMP and GTR or not.

The objective of this study was to investigate the effect of EMP at the early stage of wound healing in the periodontal tissues by a combination treatment with GTR in dogs.

MATERIALS AND METHODS

Six young, healthy male beagle dogs were used in this study. The animals were placed under general anesthesia with ketamine hydrochloride (KETALAR®) at a dosage of 10 mg/kg. In order to reduce haemorrhagia in surgical areas, a local infiltration anesthesia with lidocaine (Xylocaine®, 1 : 80,000 epinephrine) was also performed. All experiments were reviewed and approved by Guidelines for the Treatment of Experimental Animals at the Tokyo Dental College.

Following intracrevicular incisions, full thickness mucoperiosteal flaps were elevated at both the vestibular and oral aspects of the jaws. Intrabony periodontal defects at a depth of approximately 6–8 mm (measured from the cement enamel junction) were produced using a slowly rotating cylindrical bur at the distal surfaces of both the mandibular third and fourth teeth. To prevent spontaneous healing, a rubber base impression material was placed into each defect. In order to allow coronal displacement of the flaps, vertical and horizontal releasing incisions were performed. Following wound rinsing with sterile saline, the flaps were sutured with 4–0 silk strand. 4 weeks after the surgery, the rubber base impression material was removed from each defect and oral hygiene measures were initiated, which consisted of tooth brushing twice per week and topical application of 0.2% chlorhexidine. At 8 weeks following the surgery, full thickness mucoperiosteal flaps were raised, the granulation tissue was removed and root surfaces facing the defects were scaled and planed. Using a small round bur, reference notches indicating the bottoms of the defects were prepared on the root surfaces. On one side of the mandible (the experimental group), phosphoric acid gel (36%) was applied on the root surfaces for 15 s. The acid was removed by flushing the roots with sterile saline. Subsequently, a gel of EMP (EMDOGAIN®; BIORA AB, Malmö, Sweden) was applied to cover all exposed root surfaces. Following the gel application, a sample of Gore Tex Periodontal Material (GORE-TEX®; W.L.GORE & Associates, Inc., Flagstaff, Arizona, USA) was used to cover the buccal bone defect. The flaps were repositioned to cover the barrier and were sutured. The contralateral premolar (the control group) received the same treatment, but acid etching was not performed and EMDOGAIN® was not applied prior to installation of the barrier. The animals received 250 mg of streptomycin sulfate (MYCILLIN®; ZENOAQ, Fukushima, Japan), 2 times daily, for 7 days. A plaque control regimen including daily tooth cleaning was initiated and continued until the end of the experiment. The animals were euthanized with an intravenous injection of overdose of sodium pentobarbital (NEMBUTAL®; Dainihonseiyaku, Osaka, Japan) at one, two, four and eight weeks after treatment. The jaw of each animal was removed, and specimens containing the experimental tissues were placed in 10% buffered formalin. Specimens were decalcified with 10% ethylenediamine tetraacetic acid (EDTA) (Wako, Tokyo, Japan). The specimens were then dehydrated in ethanol, embedded in paraffin, serially sectioned (at 5 μm thickness) in a buccolingual orientation, and then stained with hematoxylin and eosin. From each root, 5 sections were used for microscopic examination and histometric assessment at 40 times magnification. The following linear distances were measured (Fig. 1).

1. The apical border of the notch (N) to the ce-
Effects of EMP with GTR in dogs

1. The cement enamel junction (CEJ): i.e., the height of the defect, expressed as a percentage of the height of the defect.

2. The apical border of the notch (N) to the coronal level of the newly formed cementum (C): i.e., the amount of cementum regrowth, expressed as a percentage of the height of the defect.

3. To the coronal level of newly formed alveolar bone (B): i.e., the amount of bone regrowth, expressed as a percentage of the height of the defect.

Statistical analysis was performed using the multiple comparison Scheffe’s tests with each individual animal serving as a statistical unit.

Immunohistochemistry. For immunohistochemical analysis, paraffin sections (approximately 5 μm thick) were cut in the same manner as described above and were incubated in 0.3% hydrogen peroxide for 30 min at room temperature to block endogenous peroxidase activity, after which the sections were rinsed with phosphate buffered saline (PBS) for 5 min. For antigen retrieval, sections were treated with 0.1% trypsin for 30 min at room temperature and were processed using an immunoperoxidase staining kit (Histofine SAB-PO (M) kits; Nichirei, Tokyo, Japan). The sections were incubated with mouse anti-proliferating cell nuclear antigen (PCNA) primary antibody (PC-10; DAKO Corporation Carpinteria, CA, USA) at a dilution of 1:100 for 12 h at 4°C. The sections were then incubated with biotinylated secondary antibody and streptavidin peroxidase reagents for 60 min each. The presence of peroxidase complexes was visualized by 3-3’-diaminobenzidine tetrahydrochloride (0.1 mg/ml) solution with 0.65% H2O2 for 5 min. Sections were counterstained with Mayer’s hematoxylin.

Histometric analysis. Histometric analyses were per-
Fig. 3  Microphotograph of a defect area at one week (experimental group). The morphology of the defect area is filled with active connective tissue formation. Note the dense connective tissue rich in collagen and cellularity staining for H-E (bar: 240 µm).

Fig. 4  Microphotograph of a defect area at two weeks (experimental group). The morphology of the defect area is filled primarily with dense connective tissue and newly formed bone. Note the areas of the initial bone formation located parallel to the root surface staining for H-E (bar: 240 µm).

Fig. 5  Microphotograph of a defect area at 4 weeks (experimental group). The morphology of the defect area is filled with the formation of new bone and new cementum. Note the wide marrow spaces within the trabeculae structure staining for H-E (bar: 240 µm).

Fig. 6  Higher magnification of the defect shown in Fig. 5. The newly formed cementum (arrows) is observed on the root surface (bar: 60 µm).
formed microscopically at a magnification of 400 times, after the specimens were randomly numbered such that the examiner did not know their identity. The area of connective tissue immediately beneath the coronal of the expanded polytetrafluoroethylene (e-PTFE) membrane (zone I), the central area (zone II) and the apical area (zone III) were selected in each section (Fig. 2). Each field submitted to quantitative analysis was 0.04 mm\(^2\) (0.2 mm \(\times\) 0.2 mm). In the affected areas, the numbers of PCNA-positive cells were calculated as the total value obtained from zones I, II and III. Analysis of variance and the multiple comparisons Scheffe’s test were used to analyze the data.

RESULTS
At one week after treatment, new bone or cementum formation was not observed in either group. In the experimental group, the defect area was characterized by active formation of fibrous connective tissue (Fig. 3). As the connective tissue continued to form, it gained not only thickness, but also collagen density and cellularity. In the newly produced connective tissue, there was a prominent proliferation of fibroblast-like cells (Fig. 3). In the control group, the defect area was filled primarily with granulation tissue that was composed of numerous infiltrating inflammatory cells and blood vessels. A small amount of newly formed fibrous connective tissue was found on the root as well as on the alveolar bone surface. Newly formed fibrous tissue revealed an intense cellularity in which fibroblast-like cells were interspersed.

After 2 weeks, the experimental group had a greater area of new bone and cementum formation when compared to the control group (Table 1). The mean values for new bone formation gain in the experimental group and in the control group at 2 weeks were 19.7% and 9.3%, respectively, differences that were statistically significant (P < 0.05) (Table 1). New cementum formation at 2 weeks was 24.9% in the experimental group and was only 10.0% in the control group. There was a statistically significant difference between two groups (P < 0.05) (Table 1). In the experimental group after 2 weeks, specimens demonstrating early signs of new bone formation were also evident, especially in the lateral and apical portions of the lesion (Fig. 4). In general, bone formation advanced parallel to the root surface. Further, numerous fibroblasts were located in the connective tissue adjacent to osteoblasts covering the surface of the bone trabeculae (Fig. 4). In the control group after 2 weeks, the defects were filled with fibrous connective tissue. Bone formation was observed only apically, deep in the connective tissue close to the bone. In those areas, there were many fibroblasts and rich vascularization.

After 4 weeks, statistical analysis of the data demonstrated significant differences favoring the experimental group with respect to increases of new bone and cementum formation (Table 1). The mean values for new bone formation gain in the experimental group and in the control group at 4 weeks

| Table 1 | New cementum and new bone formation following treatment of intrabony bone defects of the experimental and control groups. |
|---|---|---|---|
| | Experimental group | Control group |
| | mean ± SE (%) | mean ± SE (%) |
| New Cementum | | |
| 2 weeks | 24.9 ± 0.1 | 10.0 ± 0.01 |
| 4 weeks | 84.0 ± 0.1 | 57.2 ± 0.1 |
| 8 weeks | 99.0 ± 0.1 | 95.9 ± 0.1 |
| New Bone | | |
| 2 weeks | 19.7 ± 0.1 | 9.3 ± 0.1 |
| 4 weeks | 77.6 ± 0.2 | 55.2 ± 0.1 |
| 8 weeks | 98.3 ± 0.04 | 90.1 ± 0.12 |
| *statistically significant: P < 0.05  (n = 5)  (Scheffe’s test) |
| NS: no significant |
were 77.6% and 55.2%, respectively, differences which were statistically different (P < 0.05). A statistically significant increase was found in new cementum formation at 4 weeks of 84.0% in the experimental group compared to only a 57.2% gain in the control group (P < 0.05) (Table 1). In the experimental group, these specimens demonstrated more advanced bone formation. The newly formed bone had wide marrow spaces within the trabecula (Fig. 5). The new cementum was formed at the root surface, and the new periodontal ligament was formed between the root surface and the new bone (Fig. 6). In the control group, histological analyses identified periodontal defects below the barrier membrane, which had filled with soft connective tissue. New bone was present in the apical third of the defect, whereas the coronal portion was characterized by the presence of fibrous connective tissue. Newly formed cementum was present in the notch of the root surface.

After 8 weeks, new bone and cementum formation had occurred in the experimental and control groups, as summarized in Table 1. In the experimental group, the new bone and new cementum gains were 98.3% and 99.0%, respectively, whereas in the control group, the new bone and new cementum gains were 90.1% and 95.9%, respectively (Table 1). No statistically significant differences were found between the two different treatments with respect to new bone or cementum formation (Table 1). Qualitative histometric findings for the experimental group showed no statistically significant differences in new periodontal regeneration area compared to the control group (Table 1). In the experimental and control groups, the periodontal regeneration of the defect areas was nearly completed by further fibrous connective tissue in the defects with new bone and periodontal ligament after both types of treatments (Fig. 7). Newly formed periodontal ligament space was observed between the teeth and newly formed bone (Fig. 8). In those areas of the root where both cementum and bone had been formed during healing, a functionally oriented periodontal ligament was reestablished (Fig. 8).

**Immunohistochemistry**

Results indicating the number of PCNA-positive cells in the newly formed connective tissue under the membrane of the experimental and control groups are summarized in Table 2. At 1 and 2 weeks post-surgery, PCNA-positive cell ratios in the experimental group were significantly higher than that in the control group. PCNA-positive cell ratios in the experimental and control groups after one week were 23.1% and 11.9%, and they were 29.1% and 11.5% after two weeks, respectively, differences of those were significant (P < 0.05). At 4 and 8 weeks, no statistically significant differences were found between the two different treatments with respect to the number of PCNA-positive cells (Table 2). Results of staining for PCNA are shown in Fig. 9. The newly formed connective tissue in the experimental group showed more numerous PCNA-positive cells (brown stained nuclei) than that in the control group (Fig. 9).

**DISCUSSION**

This study evaluated the effects of EMP on GTR-induced healing of periodontal tissues in defective bones. Examination of randomly selected tissues characterized the effects of treatments with GTR.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Density (%) of PCNA-positive cells in the newly formed connective tissue of the experimental and control groups.</th>
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<tr>
<td></td>
<td>Experimental group</td>
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<td></td>
<td>Mean ± SE (%)</td>
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<tr>
<td>1 week</td>
<td>23.1 ± 0.1</td>
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<tr>
<td>2 weeks</td>
<td>29.0 ± 0.1</td>
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<td>PCNA-positive cells</td>
<td>4 weeks</td>
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<td>8 weeks</td>
<td>12.0 ± 0.2</td>
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*statistically significant: P < 0.05 (n = 5) (Scheffe’s test)
NS: no significant
alone or with GTR + EMP, based on wound healing in the same animals, all of which underwent the same experimental procedures. Recent studies on EMP have revealed that it acts on cells from mainly periodontal ligament in the early stage of maturation in wound healing (5, 10, 19). Within the limits of our knowledge, the effects of EMP at the early stage (at 1, 2, and 4 weeks) of periodontal tissue healing have not been previously investigated \textit{in vivo}. In contrast, the late stage (at 2, 3 or 5 months) of periodontal tissue healing following treatment of periodontal bone defects with GTR, EMP or GTR + EMP has been investigated (1, 20, 22).

In this study, the amount of new cementum seen at 2 and 4 weeks after treatment was significantly higher in the tissue treated with EMP and GTR than that treated with GTR alone. In the tissue treated with EMP and GTR, the ratio of new bone was accounted for 19.7% and 77.6% at 2 and 4 weeks after treatment, respectively, and that of new cementum was significantly increased in tissues treated with GTR + EMP, compared to that treated with GTR alone. In our study, proliferation of periodontal ligament cells in defective bones treated with GTR + EMP or GTR alone were compared. One and 2 weeks after treatment with EMP + GTR, PCNA-positive cells ratio was accounted for 23.1% and 29%, respectively, and a significant difference was definite between tissues treated with EMP + GTR and with GTR alone ($P < 0.05$). Based on this finding, we conclude that periodontal ligament cells react with EMP in the early stage of healing (at weeks 1, 2 and 4). EMP might stimulate cells from mainly periodontal ligament during wound healing by a mechanism different from the controls, or cells of periodontal ligament might be more sensitive of this stimulation than gingival fibroblasts. Cattaneo et al. (5) demonstrated a cell proliferative effect of EMP on human periodontal ligament cells, showing that EMP affects cell proliferation in human periodontal ligaments. They found that in an \textit{in vitro} healing model, EMP treatment affected wound filling in cultured cells (PDL, GF and MG63) compared to the control group. Furthermore, they showed that only periodontal ligament cells in the early stage of wound healing react with EMP. It has been also reported that EMP stimulates cell proliferation and acts on the early stage at days 7 and 10 of osteoblast maturation (10, 19). These previous studies support our results that EMP acts a significant rate in the early stage of wound healing in the periodontal ligament cells.

No significant differences were observed in the
Fig. 9  Immunohistochemical findings for PCNA in the experimental group. PCNA-positive cells are observed in the newly formed connective tissues, staining for SAB and counterstained with Mayer’s hematoxylin, at two weeks post-surgery (bar: 60 µm).

rates of new cementum or bone formation at week 8 between the groups (EMP + GTR: 99.0% and 98.3%, GTR alone: 95.9% and 90.1%), suggesting that in the late stage of healing (at week 8), the wound healing environment on cells of periodontal ligament mainly stimulated the healing activity of the cells, and endogenous factors generated in the wound healing environment, which have a greater influence than EMP, affected the cells. Moreover, EMP may decrease or degenerate within several weeks of treatment, and may not influence on periodontal ligament cells in the late stage of wound healing. Gestrelius et al. (10) investigated the dynamics of EMP, and found that EMP was absorbed by hydroxyapatite and by collagen, and was not retained in the root surface after week 2. An investigation by Araújo and Lindhe indicated that EMP did not affect cementum formation in the late stage of periodontal tissue healing based on the finding of the absence of EMP on the root surface in the late stage (1). This finding suggests that EMP acts on the early stage of periodontal tissue wound healing and promotes the proliferation of periodontal ligament cells.

In this study, treatment with GTR alone or treatment with GTR + EMP allowed highly predictive periodontal regeneration in defective periodontal bones. There were no obvious differences between new cementum and bone formation of the groups at week 8. This finding shows that GTR alone regenerates the periodontal tissue, showing its potential as a therapeutic method, while the amount of regenerated tissue after the combination therapy with EMP + GTR was not superior to that after GTR alone, showing that the combination therapy might not be appropriate. However, since the findings were obtained in a single animal species and in only one type of bone defect, further investigation may be necessary.

In conclusion, this study supports the cell proliferative activity of EMP on periodontal ligament cells. This study also shows that this effect is exhibited in the early stage of wound healing during the proliferation of periodontal ligament cells.

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REFERENCES


