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Hard Tissue Formation by Human Periodontal Ligament Fibroblast Cells Treated with an Emdogain®-Derived Oligopeptide in vitro

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Abstract : Periodontitis is an inflammatory disease that leads to the progressive degeneration of the alveolar bone that surrounds and supports the teeth. Without effective treatment, periodontitis can cause tooth loss. One method of treating periodontitis is to use Emdogain®, a material derived from the tooth germ of juvenile swine that promotes periodontal tissue regeneration, including the formation of hard tissue as cementum, alveolar bone. The use of Emdogain® is therefore established in the field of periodontal regenerative therapy. However, because of its swine origin, some patients choose not to be treated with Emdogain®. The active component of Emdogain® has been shown to be a peptide whose sequence corresponds to an amelogenin II precursor. As such, this peptide may function as a growth factor to stimulate cell differentiation and tissue regeneration. In this study, we characterized the effects of the synthetic Emdogain®-derived peptide on the proliferation, adhesion, migration and differentiation of periodontal ligament fibroblasts (HPdLFs), which display properties similar to mesenchymal stem cells. Compared to cells not treated with the synthetic Emdogain®-derived peptide, treated cells showed increased proliferation, initial adhesion, and chemotactic activity. The optimum peptide concentration that stimulated these activities was determined to be 100 ng/ml. We next investigated the effects of the peptide at a concentration of 100 ng/ml on osteogenesis and cementogenesis in HPdLF cells by assaying alkaline phosphatase activity, osteocalcin production, and mineralization. Compared with untreated cells, cells incubated with the peptide showed increased alkaline phosphatase activity after 21 days, increased osteocalcin production after 28 days, and increased calcium deposition after 28 days. Taken together, our data suggest that the Emdogain®-derived peptide stimulates periodontal hard tissue regeneration by stimulating the proliferation, adhesion, and migration of mesenchymal stem cells.

Keywords: Emdogain®, Periodontal ligament, Mesenchymal stem cell, Hard tissue regeneration

Introduction

Emdogain® (EMD) 1) is a material extracted from the tooth germ of juvenile swine that can generate acellular cementum and facilitate periodontal regeneration in patients with periodontitis characterized by marked alveolar bone resorption 2-3). Emdogain® or Emdogain® gel has been clinically used for more than a decade to produce marked regenerative effects 4-6). The regeneration of alveolar bone after application of EMD can be observed by X-ray analysis. In addition, the molecular mechanisms underlying the action of EMD on the structural cells of periodontal tissue have been comprehensively investigated for about a decade to substantiate its regenerative effects 7-9). Periodontal regenerative therapy with EMD is being established. The therapy is recognized as “advanced medicine” in Japan.

Because EMD is a biological material extracted from the tooth germ of juvenile swine, patients sometimes reject EMD treatment due to concerns about unknown pathogens. Currently, many patients still choose guided tissue regeneration (GTR) with a non-resorbable or resorbable membrane, an autogenous graft or a combination of these methods for periodontal regenerative therapy in Japan 10-12). Therefore, to best utilize the clinical data obtained regarding the regenerative effects and postoperative stability of EMD, EMD-derived synthetic peptides should be developed. Traditional EMD (Emdogain®) was subcutaneously injected into the backs of rats to form eosinophilic corpuscles and chondroid tissue 13), and MALDI-TOF analysis of the eosinophilic corpuscles
demonstrated that the fragments contained the amino acid sequence, WYQNMIR. Database analysis demonstrated that the sequence corresponded to a swine amelogenin II precursor. A peptide based on the sequence was synthesized and subcutaneously inoculated into the backs of rats. Two weeks later, the peptide induced bone, cartilage and endochondral ossification, suggesting that it induced hard tissue formation \(^{14}\). Specifically, the peptide may function as a growth factor to induce cell differentiation for tissue engineering applications \(^{15}\). The activity of the peptide should therefore be investigated both in vitro and in vivo.

To investigate the functions of novel growth factors, their optimal concentrations should be determined in vitro and in vivo. Various growth factors used for regenerative therapy, such as platelet-rich plasma (PRP), platelet-derived growth factor and basic fibroblast growth factor (b-FGF), have been investigated and applied clinically. Graziani et al. examined the effects of PRP on osteoblasts and fibroblasts, and demonstrated that the effects of PRP were dose-dependent and that increased doses were not necessarily effective for tissue regeneration \(^{16}\). Gamal et al. examined the effects of PDGF-BB on the adhesion of periodontal ligament fibroblasts obtained from a patient with periodontitis. PDGF-BB was most active at 50 ng/mL \(^{17}\). However, the effects remained the same at 50 ng/mL or above. Thus, 50 ng/mL was determined to be the optimal dose for treating periodontal wounds.

Takayama et al. demonstrated that the effects of b-FGF on human periodontal ligament cell proliferation were dose-dependent, and that b-FGF stimulated higher ALP activity at lower concentrations during differentiation of the cells into calcified tissue \(^{18}\). Thus, there may be optimal concentrations for the biological application of growth factors. In particular, in vitro studies demonstrate that the kinetics of growth factors vary according to the sources of cells.

In this study, we examined the effects of the novel synthetic peptide derived from EMD on human gingival epithelial cells \(^{19}\). The peptide was effective in suppressing the proliferation of epithelial tissue immediately after periodontal surgery until periodontal regeneration, and in facilitating postoperative wound healing. We previously reported that the peptide induced osteogenesis \(^{20}\) and cementogenesis \(^{21}\) in animal experiments using SD rats, and stimulated the differentiation of rat bone marrow cells into hard tissue in vitro. According to GTR principles, inhibition of both the growth of epithelial cells and the proliferation of mesenchymal stem cells are required in periodontal regeneration therapy to make space for a regenerative region and strong connective tissue adhesion, instead of long epithelial adhesion \(^{22-25}\).

The optimal concentration of the novel \(^{13}\) EMD-derived synthetic peptide remains unknown. Here, we used human periodontal ligament fibroblasts (HPdLF) as a model of mesenchymal stem cells to determine the optimal concentration of the peptide required to stimulate their proliferation, adhesion and migration. The effects of the optimal concentration of the peptide on hard tissue formation like cementum or alveolar bone were then examined.

**Material and Methods**

**Cell culture**

The HPdLF cells used in this study were purchased from Lonza. HPdLF cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Wako Pure Chemical Industries, Ltd, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Life Technologies Corp., Carlsbad, CA, USA), penicillin (500 U/mL), streptomycin (500 µg/mL), and fungizone (1.25 µg/mL) (Cambrex Bio Science Walkersville Inc.) in 75 cm² culture flasks (Falcon, Becton Dickinson Labware, NJ, USA). The cells were cultured at 37°C in a humidified 5% CO₂/95% air atmosphere. Upon reaching confluence, the cells were detached from the flasks by trypsinization and washed twice in PBS, resuspended in culture medium and seeded. The cells from passage 4–6 were used in the following experiments.

**Peptide synthesis**

The oligopeptide, WYQNMIR, derived from Emdogain® was prepared by traditional solid-phase peptide synthesis in conjunction with “tea-bag” methodology using Boc/benzyl-based chemistry. Briefly, chondrogenesis was observed 7 days after injection of 30 mg of EMD dissolved in 1 mL of propylene glycol into the backs of rats, with numerous eosinophilic round bodies (ERBs) around the formed cartilage tissue. ERBs were examined in pathological sections and analyzed biochemically. ERBs were pathologically found not to contain polysaccharide, amyloid or hemosiderin. Biochemical analysis of ERBs revealed a 40-kd band that did not contain EMD. Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and database analysis of ERBs identified a seven-amino-acid sequence encoded by amelogenin-exon 5.

**Cell proliferation assay**

HPdLF cells were harvested and plated at a density of 4×10⁴ cells/well in 96-well microplates (Falcon). After 24 hours, the medium was replaced with medium containing the synthetic oligopeptide at concentrations of 0, 5, 20, 100, 200 or 500 ng/mL. After washing the microplates with PBS, cell proliferation/viability was determined using the CellTiter-Blue™ Cell Viability Assay Kit (Promega, Madison, WI, USA) after 1, 3, 6 and 72 h of incubation according to manufacturer’s instructions. Initially, the CellTiter-Blue™ reagent was diluted 6 times in PBS. Following aspiration of the medium and washing with PBS, 100 µL of reagent were added to each well and the plates were incubated for 1 h at 37°C in a humidified 5% CO₂/95% air atmosphere. Fluorescence
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intensity (excitation 560nm, emission 590nm) was measured using a multi-microtiter reader (SpectraMax® M5, Molecular Device Inc, Sunnyvale, CA, USA).

**Cell adhesion assay**

Cell adhesion assays were performed as described previously. 96 well enzyme-linked immunosorbent assay (ELISA) plates (Nalgene Nunc, Thermo Fisher, Denmark) were coated with the synthetic oligopeptide at concentrations of 0, 5, 20, 100, 200 or 500 ng/ml (50 μl per well) and incubated at 37°C for 3 h. The peptide was then removed and the plates were blocked with 1.2% BSA (Sigma, St. Louis, MO, USA) in PBS (200 μl per well) for 1 h at 37°C.

HPdLF cells were harvested and plated at a density of 2×10^4 cells/well, and the plates were incubated at 37°C for 1 h. Subsequently, the plate was shaken for 5 seconds and the cells were fixed by adding 150 μl of 4% paraformaldehyde in phosphate buffer (Nacalai Tesque Inc, Kyoto, Japan) to each well and incubating for 10 min at room temperature. The attached cells were fixed and stained with 0.25% PicoGreen® ds DNA solution (Invitrogen, Life Technologies Corp., Carlsbad, CA, USA) for 5 min. The plates were read on an ELISA plate reader (SpectraMax® M5) at an excitation wavelength of 450 nm and an emission wavelength of 530 nm.

**Cell chemotaxis assay**

To investigate the chemotaxis of HPdLF cells, a modified Boyden chamber assay was performed using a 24-well microchemotaxis chamber (Fluoroblock Insert system; Falcon) as previously described. Equal numbers of HPdLF cells were suspended and preloaded with the non-toxic fluorescent indicator Calcein AM (4 μM; Molecular Probes, Inc., Oregon, USA) and incubated for 30 min at 37°C. Cells were trypsinsized (0.5% trypsin, 5 min), washed in medium, harvested and seeded at a density of 4×10^3 cells/well in 24-well microplates (Falcon) and resuspended in culture medium without FBS (Wako, Osaka, Japan) to yield a final cell concentration of approximately 2×10^4 cells/500 μl cell suspension was added to each cell culture insert (upper chamber). Next, 750 μl of medium containing the synthetic peptide at 5, 20, 100, 200 or 500 ng/ml, and medium with or without FBS as positive and negative controls, respectively, were placed in the wells of the lower chamber plate by carefully pipetting between the walls of the upper and lower chambers.

The contents of the upper and lower wells were separated by a 3.0 μm pore size HTS FluroBlock™ Insert (Falcon). Cell chemotaxis was observed for 1 h, 3 h and 8 h at 37°C in a humidified 5% CO₂,95% air atmosphere. HPdLF cells that had passed through the filter into the lower chamber were evaluated on a fluorescence plate reader (SpectraMax® M5) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.
The effect of the synthetic oligopeptide on the proliferation of HPdLF cells as measured by MTS assay is shown in Fig. 1. After incubation with the peptide for 1 and 3 hours, the fluorescence intensity was highest in the 100 ng/ml oligopeptide group. After incubation for 6 hours, there was no significant difference in the fluorescence intensity between the 100 ng/ml oligopeptide group and the group incubated in medium without oligopeptide. After 6 hours of incubation, the fluorescence intensities in the 20 ng/ml and 100 ng/ml oligopeptide groups were significantly higher than in the other groups.

**Effect of the Emdogain®-derived synthetic oligopeptide on the adhesion of HPdLF cells**

Fig. 2 shows that cell adhesion was highest in the group incubated with 100 ng/ml of oligopeptide at 30 s and 3 h after cell plating. Cell adhesion in the 20 ng/ml and 100 ng/ml oligopeptide groups was significantly higher than in the other groups at 2 h after cell plating. After incubation for 1 hour, there was no significant difference in cell adhesion between the 100 ng/ml oligopeptide group and the group incubated in medium without oligopeptide.

**Effect of the Emdogain®-derived synthetic oligopeptide on the chemotaxis of HPdLF cells**

HPdLF cells were stained with CalceinAM. Because the fluorescence lifetime of CalceinAM is approximately 8 hours, fluorescence was measured at 1, 4, 6 and 8 hours after the beginning of the HPdLF cell chemotaxis assays. The effect of the synthetic oligopeptide on the chemotaxis of HPdLF cells measured by the modified Boyden chamber method is shown in Fig. 3. Cell chemotaxis was significantly greater in the positive control group (medium containing FBS) than in the negative control group (medium not containing FBS) at all time points. After 1, 4, 6 and 8 hours of incubation, chemotaxis in the 100 ng/ml oligopeptide group was significantly higher than in the group incubated in medium without oligopeptide.

**ALP activity**

100 ng/ml of oligopeptide was determined as the optimal concentration for differentiation studies. Cell differentiation was assessed by measuring the activity of the differentiation marker, ALP, in the test and control groups after incubation with the oligopeptide for 21 days. At this point, ALP activity was significantly higher in the cells of the test group compared with the control group (Fig. 4).

**Osteocalcin Production**

Fig. 5 shows the production of OCN in the test and control groups after incubation with the oligopeptide (100 ng/ml) for 28 days. The presence of OCN in the supernatant of the test group was significantly higher than in the control group.

**Mineralization**

Fig. 6 shows calcium deposition in the extracelluar matrix of HPdLF cells in the test and control groups after incubation with the oligopeptide (100 ng/ml) for 28 days. The deposition of calcium is cumulative such that the levels measured increase with exposure time. Significantly more calcium was deposited by the cells in the test group than by cells in the control group after 28 days.

**Discussion**

In this study, we examined the effects of an Emdogain®-derived synthetic peptide on the proliferation and differentiation of HPdLF cells as a model of mesenchymal cells. First, HPdLF cell proliferation, adhesion and migration were examined at five peptide concentrations to determine the optimal concentration of the peptide. Next, the differentiation of HPdLF cells into hard tissue after stimulation with the optimal concentration of peptide was examined. Because stimulation with 100 ng/mL of peptide resulted in the highest proliferation, adhesion and migration activities of the HPdLF cells, 100 ng/mL was determined to be the optimal concentration for the differentiation studies. Incubation of HPdLF cells with 100 ng/mL of peptide significantly increased ALP activity after 14 and 21 days, osteocalcin production in the culture supernatant after 21 days, and intracellular calcium deposition levels after 28 days, in the experimental group compared with the control group.

Periodontal growth and regeneration require novel periodontal tissue formation, including cementum, alveolar bone and periodontal ligament, due to cell growth in intraosseous defects after inflammatory tissue dissection. The most rapidly growing tissue during early wound healing after periodontal surgery is gingival epithelium, which forms a gingival full-thickness flap. Epithelial cells proliferate to facilitate apical growth deep into the epithelial tissue and form long epithelial junctional adhesions parallel to the tooth roots. However, this healing does not promote new connective tissue and precludes periodontal regeneration 26). Kramer suggested that the suppression of deep epithelial proliferation prior to the formation of new periodontal tissue is required to achieve regeneration during early healing 27). Because regeneration materials such as Emdogain®, PRP and PDGF act as growth factors to stimulate various cells and induce regeneration 28), we suggest that the Emdogain®-derived synthetic peptide also functions as a growth factor. Early proliferation and differentiation of mesenchymal cells, i.e., periodontal ligament cells or bone marrow mesenchymal cells, is required to repair periodontal tissue defects 19). In particular, immediately after application of regeneration materials, an increased number of cells are required to suppress deep epithelial proliferation and the formation of long epithelial adhesions rather than to differentiate into hard tissue. Thus, we hypothesized that cell proliferation, adhesion and
Figure 1. Effect of the synthetic oligopeptide on the proliferation of HPdLF cells measured by MTS assay. After 1 and 3 hours of incubation with the oligopeptide, the fluorescence intensity in the 100 ng/ml oligopeptide group was higher than in the groups treated with the other concentrations of the oligopeptide. After 6 hours of incubation, there was no significant difference in the fluorescence intensity between the 100 ng/ml oligopeptide group and the group incubated in medium without oligopeptide. After 72 hours, the fluorescence intensities in the groups incubated with 20 ng/ml and 100 ng/ml of oligopeptide were significantly higher than in the other groups (*: \( P < 0.05 \)).

Migration should be assayed to determine the optimal concentration of the peptide for use in differentiation studies.

Melcher suggested that the main goal of periodontal regenerative therapy is to induce connective tissue adhesion, and cell adhesion to tooth roots plays an important role during wound healing after periodontal surgery. We regard adhesion of the periodontal ligament-oriented cells into the root surface as important. Adhesions between periodontal ligament cells and the root surface, or the cells and the extracellular matrix, are necessary and indispensable for the formation of connective tissue. The adhesion of periodontal ligament cells to extracellular matrix components including collagen and fibronectin is thought to influence the chemotaxis, growth and differentiation of periodontal cells. Monique et al. reported that Emdogain® acts as a growth factor to mediate this adhesion. Therefore, that Emdogain® increases the adhesion of human periodontal ligament cells agrees with our results showing the increased adhesion of human periodontal ligament cells to plates coated with 100 ng/ml of the synthetic Emdogain® peptide after 30 minutes, 2 hours or 3 hours of incubation.
Many reports have described how Emdogain® functions as a growth factor that promotes the proliferation of HPdLF cells. Nagano et al. reported that the increased proliferation of periodontal ligament cells is effective for periodontal regenerative therapy. However, Zhaman et al. reported that osseous ankylosis may be inhibited by the growth rate of periodontal ligament cells, which is faster than the growth rate of cells derived from alveolar bone. This is caused by the fact that periodontal ligament cells consist of a heterogeneous cell population including undifferentiated mesenchymal cells. Periodontal ligament cells have been suggested to differentiate into various different cell types. The synthetic Emdogain® peptide promoted the growth of periodontal ligament cells at each time point tested, with a concentration of 100 ng/ml stimulating the highest rate of proliferation. It is thought that the proliferation rate of HPdLF cells is high prior to their differentiation into cementoblasts and osteoblasts. Also, our results agree with the report by Hoang et al., which showed that HPdLF cells proliferate after the formation of a cement-like hard tissue by Emdogain®.

Rincon et al. suggested that periodontal ligament cell migration, as well as adhesion and proliferation, is increased by Emdogain®. Because alimentation to the regenerative area is necessary for enabling periodontal tissue to regenerate, the activity of Emdogain® as a regenerative material is strongly associated with...
the increased migration of periodontal ligament cells. Our results suggest that the Emdogain®-derived synthetic peptide initiates cell attachment, which then leads to cell proliferation and chemotaxis. The potential for hard tissue formation was examined at the optimal concentration of 100 ng/ml, which was determined from the results of the HPdLF cell attachment, proliferation and chemotaxis experiments.

Many reports have been published on the differentiation of HPdLF cells into bone. Our results suggest that Emdogain® contributes to increased ALP activity, but Hoang et al. reported that HPdLF cells exhibit multipotent differentiation similar to mesenchymal stem cells and may form cementum [34]. Nagano et al. reported that HPdLF cells can differentiate into osteoblastic cells [32, 35 - 37]. The Emdogain®-derived synthetic peptide promoted increased ALP activity in this study. Additional studies will be necessary in future, but our results suggest that this peptide contributes to the formation of cementum as reported by Noguchi et al., and this observation agrees with the report of Hoang et. al [34].

In this study, OCN production and calcium deposition were increased by the Emdogain®-derived synthetic peptide. Many studies have shown that Emdogain®, which is extracted from pig juvenile enamel, leads to bone and cementum formation [32, 35 - 37]. Alveolar bone and cementum are both required for periodontal regeneration, and are essential for the maintenance of hard tissue morphology [31]. Nagano et al. reported that the expression of genes encoding ALP, osteopontin, osteocalcin, BSP and cementum is increased in HPdLF cells, suggesting that these cells differentiate into osteoblastic cells or cementum [70]. The results from studies...
by Nagano et al.\textsuperscript{29,30} and Monique et al.\textsuperscript{29,30}, which showed that Emdogain® increases OCN production and calcium deposition, suggest that calcification is accelerated by Emdogain®. The Emdogain®-derived synthetic peptide used in this study also accelerated calcification, suggesting that this peptide makes bone or cementum.

This study suggests that an Emdogain®-derived synthetic peptide facilitated hard tissue formation during periodontal regeneration by stimulating the proliferation, adhesion and migration of HPdLF cells. Further studies are required to determine which cells HPdLF cells differentiate into, and what effects the Emdogain®-derived synthetic peptide has on other periodontal cells.

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