A Human Amelogenin-Derived Oligopeptide Enhances Osteogenic Differentiation of Human Periodontal Ligament Stem Cells

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Abstract: We have found newly synthesized peptide derived from commercially available Emdogain® for periodontal tissue regeneration therapies in our previous study. That synthesized peptide consisted of seven amino acid sequence, WYQNMIR, which is exon 5 of porcine amelogenin and had the same effects as Emdogain®. The amino acid sequence of human amelogenin exon 5 is WYQSIR which both N (asparagine) and M (methionine) in porcine amelogenin are replaced by S (serine). In the present study, we investigated the effect of a synthetic human amelogenin-derived peptide (HAP) consisting of WYQSIR on human periodontal ligament stem cells (PDLSCs) to be useful for tissue regeneration. PDLSCs were isolated from third molars of adult donors. The effect of HAP on PDLSCs was investigated by culturing them in normal or osteogenic medium with and without HAP. Proliferation of the PDLSCs was evaluated using a cell proliferation assay after they had been treated with HAP (ranging from 1 ng/ml to 10,000 ng/ml) for 1, 3, 5, and 7 days. Osteogenic differentiation was evaluated by measuring alkaline phosphatase (ALP) activity, calcium deposition, osteocalcin production, and mRNA expression of Runx2 and osteoectin after the PDLSCs had been treated with HAP (1,000 ng/ml) for 1, 7, 14, and 21 days. The number of viable cells was significantly increased in the presence of HAP in normal medium. Compared to cells cultured without HAP in osteogenic medium, cells cultured with HAP showed significantly increased ALP activity, calcium deposition, osteocalcin production, and Runx2 mRNA expression. The results show that HAP enhances proliferation and osteogenic differentiation of PDLSCs. The present study suggests that HAP may be a useful material for periodontal tissue regeneration.

Key words: Amelogenin, Regeneration, Osteogenesis, Periodontal ligament, Mesenchymal stem cells

Introduction

Emdogain® is the predominant protein constituent of enamel matrix derivative (EMD), which is extracted from the tooth germs of 6-month-old pigs. EMD induces the formation of acellular cementum, periodontal ligament, and alveolar bone. EMD is commonly used in periodontal tissue regeneration therapies. Although one study showed the clinical safety of EMD in the treatment of periodontal defects, the possibility that EMD may contain unknown pathogens cannot be ruled out since it is derived from animal tissue. Furthermore, EMD is antigenic and induces the production of anti-EMD antibodies in patients exposed to it. Therefore, a completely synthetic material for periodontal tissue regeneration should be developed.

In our previous study, EMD injected subcutaneously into the backs of rats induced the formation of eosinophilic round bodies (ERBs) and cartilage tissue. Biochemical analysis of these ERBs was conducted using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). The ERBs consisted of various lengths of peptide fragments, all of which contained a seven amino acid sequence (WYQNMIR). This amino acid sequence corresponds to a portion of porcine amelogenin exon 5, and a porcine amelogenin-derived peptide (PAP) was constructed based on this sequence.

We found that bone and cartilage tissue were formed subcutaneously in the backs of rats injected with PAP, and that PAP induced the formation of hard tissue and promoted early wound healing in artificial periodontal tissue defects in rats. In our previous in vitro studies, PAP promoted the proliferation and osteogenic differentiation of rat bone marrow stromal cells, human mesenchymal stem cells (MSCs), human periodontal ligament (PDL) fibroblasts, and human periodontal ligament stem cells (PDLSCs). These findings suggest that PAP is useful for periodontal tissue regeneration, and exon 5 in the sequence of amelogenin may have an important role in the process of regeneration.

The amino acid sequence of exon 5 in human amelogenin is partly different from porcine amelogenin. Although human full-length amelogenin enhances the osteogenic differentiation of human bone marrow MSCs, it has not been clarified whether human amelogenin exon 5 has any effect on the process of osteogenic differentiation. In this
study, we synthesized a human amelogenin-derived peptide (HAP). This HAP consists of a six amino acid sequence (WYQSIIR), which corresponds to a portion of human amelogenin exon 5. In general, only peptides of greater than approximately 10 residues or over 5 kDa can function as antigens\(^{16,17}\). Because the molecular mass of HAP is 852 Da, there is very little risk of eliciting an immunologic response.

PDLCs are a type of mesenchymal stem cell that exist in the periodontal ligament\(^{46}\). PDLCs have the potential to differentiate into osteoblasts\(^{19,20}\) and enhance the periodontal tissue regeneration capacity\(^2\). PDLCs exhibit greater potential in the regeneration of periodontal tissues than other mesenchymal stem cells, such as bone marrow stromal cells\(^{20}\). Therefore, PDLCs play a particularly important role in periodontal tissue regeneration. The aim of this study was to elucidate the effect of HAP on the proliferation and osteogenic differentiation of PDLCs.

Materials and Methods

**Isolation and culture of PDLCs**

Impacted and non-carious third molars were extracted from three patients (two females and a male, aged 21 to 29 years). After extraction, the teeth were rinsed in Dulbecco’s Modified Eagle’s Medium (DMEM) with 500 U/ml penicillin, 500 µg/ml streptomycin, and 1.25 µg/ml amphotericin B (all from Nacalai Tesque, Kyoto, Japan). After rinsing, the PDL tissues were separated from the middle one-third of the root surface and minced into 1-mm³ pieces. The minced tissues were digested for 1 h at 37 °C in a solution of 3 mg/ml collagenase type I (Wako Pure Chemical Industries Ltd, Tokyo, Japan) and 4 mg/ml dispase (Gibco, Thermo Fisher Scientific, Grand Island, NY, USA). The digested tissue samples were pooled, and single-cell suspensions were obtained by passing the pooled tissues through a 70-µm strainer (Falcon BD, Franklin Lakes, NJ, USA). The cells were centrifuged at 1,000 rpm for 5 min and resuspended in normal culture medium containing 10% fetal bovine serum (Biowest, Nuaille, France), 500 U/ml penicillin (Nacalai Tesque), 500 µg/ml streptomycin (Nacalai Tesque), and 1.25 µg/ml amphotericin B (Nacalai Tesque). The cells were then seeded onto T75 culture dishes (AGC Techno Glass, Shizuoka, Japan), and incubated at 37 °C in 5% CO\(_2\). The presence of single-cell colonies was confirmed after 5 to 10 days. PDLCs at passage zero (P0) were seeded, and cells at P3 to P5 were used for the experiments in the present study. PDLCs were obtained in accordance with the medical ethics guidelines of Osaka Dental University, and all experiments were approved by the Osaka Dental University Medical Ethics Committee (approval no. 110897). All participants provided written informed consent to participate in the present study, and the study design was approved by the appropriate ethics review board.

**Characterization of PDLCs**

The PDLCs were identified by immunocytochemistry, as previously described\(^{18,20}\). The PDLCs were plated at a density of 2 × 10⁴ cells/ml in 300 µl of normal culture medium on Lab-Tek® Chamber Slides (Thermo Fisher Scientific Inc, Waltham, MA, USA) and incubated for 3 days. The cells were fixed in cold 70% ethanol for 20 min at -20 °C, and blocked with 3% bovine serum albumin in phosphate-buffered saline (PBS) for 30 min at room temperature. The cells were then incubated with anti-STRO-1 antibody (Thermo Fisher Scientific Inc.), or anti-SSEA-4 antibody (Thermo Fisher Scientific Inc.) overnight at 4 °C. After washing with PBS, the cells were incubated for 60 min at room temperature with a fluorescently labeled secondary anti-mouse polyclonal immunoglobulin G antibody (Thermo Fisher Scientific Inc.). The samples were washed with PBS and mounted in Vectashield® Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Images were obtained with a LSM 700 fluorescence microscope (Carl Zeiss, Jena, Germany).

**Preparation of synthetic oligopeptide**

The synthesis of HAP was based on the amino acid sequence, WYQSIIR. HAP was prepared by traditional solid-phase peptide synthesis in conjunction with the “tea-bag” methodology using Boc/benzyl-based chemistry.

**Cell proliferation assay**

PDLCs were plated into 96-well microplates at a density of 2 × 10⁴ cells/ml in normal culture medium (100 µl/well) with and without HAP (1, 10, 100, 1,000, or 10,000 ng/ml), and the cells were cultured for 1, 3, 5, and 7 days. The number of viable cells at each time point was determined by measuring the amount of formazan generated in 6 wells per group with a formazan detection kit (Nacalai Tesque). The absorbance of formazan was measured at a wavelength of 450 nm, and the data were analyzed with the Soft Max® Pro Microplate Data Acquisition and Analysis software (Molecular Devices, Sunnyvale, CA, USA).

**Measurement of alkaline phosphatase (ALP) activity**

PDLCs were plated into 24-well microplates at a density of 4 × 10⁴ cells/ml and cultured to confluence in normal culture medium. The medium was replaced with osteogenic medium containing 50 µM L-ascorbic acid 2-phosphate (Nacalai Tesque), 10 mM β-glycerophosphate (Wako), and 10 nM dexamethasone (MP Biomedicals, LLC, Santa Ana, CA, USA) with and without HAP (1,000 ng/ml), and the cells were cultured for 7 and 14 days. The cells were washed with PBS and lysed with 0.2% Triton X-100 (Sigma, St. Louis, MO, USA). The cell lysates were treated with a 1-Step PNPP substrate (Pierce Biotechnology, Inc., Rockford, IL, USA), and the absorbance was measured at a wavelength of 405 nm. The DNA content was measured using the PicoGreen dsDNA Assay Kit (Invitrogen, Paisley, UK). To normalize the ALP activity, the amount of ALP was normalized to the amount of DNA in the cell lysate. The data were analyzed with the Soft Max® Pro software (Molecular Devices).

**Measurement of calcium deposition**

PDLCs were plated into 24-well microplates at a density of 4 × 10⁴ cells/ml and cultured to confluence in normal culture medium. The medium was replaced with osteogenic medium with and without HAP (1,000 ng/ml), and the cells were cultured for 21 days. Extracellular calcium deposition was measured after dissolving with 10% formic acid. The amount of calcium was quantified using a Calcium E-test Kit (Wako) according to the manufacturer’s protocol. The absorbance was measured at a wavelength of 610 nm, and the data were analyzed with the Soft Max® Pro software (Molecular Devices).

**Alizarin red staining**

PDLCs were plated into 24-well microplates at a density of 4 × 10⁴ cells/ml and cultured to confluence in normal culture medium. The medium was replaced with osteogenic medium with and without HAP (1,000 ng/ml), and the cells were cultured for 21 days. The cells were washed with PBS and fixed in 70% ethanol for 10 min at -20 °C. PDLCs were stained with a solution of 1% alizarin red S (Wako) for 3 min at room temperature and washed 3 times with distilled water. Pictures of the calcified nodules were processed using a microscope (Olym-
Measurement of osteocalcin

PDLSCs were plated into 24-well microplates at a density of $4 \times 10^4$ cells/ml and cultured to confluence in normal culture medium. The medium was replaced with osteogenic medium with and without HAP (1,000 ng/ml), and the cells were cultured for 21 days. The culture supernatant was collected, and the osteocalcin levels were measured with an osteocalcin detection kit (Takara Bio, Shiga, Japan).

Quantitative real-time polymerase chain reaction (PCR)

The mRNA expression levels of Runx2 and osteonectin were determined by quantitative real-time PCR analysis. PDLSCs were plated into 24-well microplates at a density of $4 \times 10^4$ cells/ml and cultured to confluence in normal culture medium. The medium was replaced with osteogenic medium with and without HAP (1,000 ng/ml), and the cells were cultured for 24 h. The total cellular RNA was extracted using an RNeasy Mini Kit (Qiagen, Venlo, the Netherlands), and 10 µl RNA from each sample was reverse transcribed into complementary DNA using a PrimeScript RT Reagent Kit (Takara Bio). All real-time PCR assays were performed according to the manufacturer’s protocol. Gene expression was calculated using a StepOnePlus Real-time PCR System (Thermo Fisher Scientific Inc.) and normalized to GAPDH expression.

Statistical analysis

In this study, 6 wells were prepared for the cell proliferation assay, and 3 wells were prepared for the remaining experiments; each experiment was repeated 3 times. Data are presented as mean ± SD, and were analyzed using SPSS Statistics Ver. 17 (IBM, Chicago, IL, USA). One-way analysis of variance followed by Tukey’s post hoc test was used to determine significance in the cell proliferation assay, and Student’s t-test was used in the remaining experiments. P values <0.05 were considered significant.

Results

Isolation and characterization of PDLSCs

The PDL-derived cells formed clonogenic clusters of fibroblast-like cells. The PDLSCs derived from the PDL cells were positive for STRO-1 (Fig. 1A) which is one of the most well-known markers for MSCs. It has been heavily relied upon for the recognition and isolation of various types of MSC, particularly those in dental tissues25). The PDLSCs were also positive for SSEA-4 (Fig. 1B), which is an embryonic stem cell marker that has also been detected in periodontal ligament-derived MSCs26).

Cell proliferation

The effect of HAP on the proliferation of PDLSCs is shown in Fig. 2. HAP significantly enhanced the proliferation of PDLSCs cultured for 1, 5, and 7 days in normal culture medium. Additionally, HAP induced
the highest cell proliferation at a concentration of 1,000 ng/ml. Therefore, 1,000 ng/ml of HAP was determined to be the optimal concentration for subsequent assays.

**ALP activity**

The intracellular ALP activity of PDLSCs was significantly enhanced in the presence of HAP on 7 and 14 days, compared with the cells cultured without HAP (Fig. 3). To normalize ALP activity, the amount of ALP was normalized to the amount of DNA. A: Day 7, *p < 0.05 vs. control. B: Day 14, *p < 0.05 vs. control.

**Mineralization**

Calcium deposition in the extracellular matrix of PDLSCs cultured in osteogenic medium for 21 days was significantly increased in the presence of HAP compared with the control (Fig. 4A). The number and size of calcified nodules stained with alizarin red S after incubation in osteogenic medium for 21 days were larger in the presence of HAP than in the control (Fig. 4B). *p < 0.05 vs. control.

**Osteocalcin production**

The production of osteocalcin in the supernatant of PDLSCs after incubation in osteogenic medium for 21 days was significantly increased in the presence of HAP compared with the control (Fig. 5). *p < 0.05 vs. control.

**Runx2 and osteonectin mRNA expression**

Runx2 mRNA expression after incubation in osteogenic medium for 24 h was significantly enhanced in the presence of HAP compared with the control (Fig. 6A). However, osteonectin mRNA expression after 24 h incubation was significantly lower in the presence of HAP than in the control (Fig. 6B).

**Discussion**

The results of this study show that HAP significantly promotes proliferation of PDLSCs in normal medium. In the osteoblast differentiation assays, ALP activity, osteocalcin production, extracellular calcium deposition, and mRNA expression of Runx2 were significantly enhanced in osteogenic medium containing HAP.

EMD stimulates proliferation of C2C12 cells (a typical pluripotent mesenchymal cell line), bone marrow stromal cells, and PDL cells27-29. In our previous studies, PAP promoted proliferation of rat bone marrow stromal cells, human MSCs, PDL fibroblasts, and PDLSCs9-11,13. In the present study, HAP promoted proliferation of PDLSCs. In particular, HAP at a concentration of 1,000 ng/ml stimulated the highest rate of cell
proliferation, whereas PAP at concentrations of 100 ng/ml or 10ng/ml had the strongest stimulatory effects in the previous studies. Therefore, the optimal concentration of HAP in PDLSCs is different from that of PAP, although HAP and PAP have similar effects in stimulating cell proliferation.

ALP is a marker of the osteoblastic phenotype that is secreted during the middle period of osteoblast differentiation and closely related to initiation of mineralization. EMD enhances ALP activity in osteoblasts and C2C12 cells. PAP promotes ALP activity in rat bone marrow stromal cells, MSAs, PDL fibroblasts, and PDLSCs. In the present study, HAP increased ALP activity in PDLSCs. Therefore, HAP stimulates the initiation of mineralization and promotes osteogenic differentiation of PDLSCs.

Osteocalcin is a non-collagenous protein component of bone matrix and a late marker of osteoblast differentiation. EMD promotes osteocalcin mRNA expression in C2C12 cells, and bovine amelogenin promotes osteocalcin mRNA expression in rat bone marrow MSCs. PAP also promotes the production of osteocalcin in MSCs, PDL fibroblasts, and PDLSCs. In the present study, HAP promoted osteocalcin production in PDLSCs. Thus, HAP seems to enhance the differentiation of PDLSCs into mature osteoblasts during the late stage of bone formation.

The quantity of calcium deposition in cultured PDLSCs was assessed by alizarin red staining and measurement of extracellular calcium deposition. Alizarin red staining is used as a biochemical mineralization assay to ascertain the presence of mineralized nodules formed by cells of osteogenic lineages; these nodules are indicative of the calcification of bone matrix. In previous studies, alizarin red staining was used to evaluate the mineralization of PDLSCs. In the present study, HAP enhanced both the number of mineralized nodules and extracellular calcium deposition in PDLSCs. These findings suggest that HAP promotes the mineralization of extracellular matrix of PDLSCs and differentiation into hard tissue-forming osteoblasts.

Runx2 is an essential factor for osteoblast differentiation. Runx2 directs the differentiation of mesenchymal stem cells into preosteoblasts, and inhibits them from differentiating into the adipocytic and chondrocytic lineages. Recent studies reported that EMD enhanced Runx2 mRNA expression in PDLSCs. In the present study, HAP enhanced Runx2 mRNA expression in PDLSCs in the early period of osteogenic differentiation. Therefore, the results of the present study suggest that HAP promotes the differentiation of PDLSCs into preosteoblasts.

Osteonectin is a glycoprotein expressed in newly differentiated osteoblasts of developing bone tissue. Bovine amelogenin promotes osteonectin mRNA expression in rat bone marrow MSCs. Although PAP promoted osteonectin mRNA expression in PDLSCs, HAP showed a negative effect on the expression of osteonectin mRNA. Thus, these results indicate that PAP and HAP promote osteogenic differentiation of PDLSCs based on different molecular mechanisms.

Human full-length amelogenin enhances cell proliferation and osteogenic differentiation of bone marrow MSCs, whereas it has no effect on PDL cells. Therefore, it is suggested that both human full-length amelogenin and HAP promote cell proliferation and osteogenic differentiation of undifferentiated MSCs, and that human amelogenin exon 5 has an important role in hard tissue regeneration.

The present study suggests that HAP has the potential to promote periodontal tissue regeneration by enhancing cell proliferation and osteogenic differentiation of PDLSCs. However, the reason for the difference in the optimal concentrations between HAP and PAP is unclear, and the detailed molecular mechanisms of the effect of HAP need to be elucidated in future studies.

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Conflict of Interest

The authors declare that there are no conflicts of interest related to this study.

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