INTRODUCTION

Emdogain® (EMD) extracted from the tooth germ of juvenile swine is a material that can generate acellular cementum and facilitate periodontal regeneration in patients with periodontitis featuring marked alveolar bone resorption. EMD has been clinically used as Emdogain® gel for more than a decade to produce marked regenerative effects. In addition, the molecular mechanisms behind the action of EMD on the structural cells of periodontal tissue have been comprehensively investigated for about a decade to substantiate its regenerative effects. Periodontal regenerative therapy with EMD is being established. The therapy is recognized as an advanced medical technology in Japan. However, EMD is a biological material extracted from the tooth germ of juvenile swine. Thus, patients reject EMD treatment because of concerns about unknown pathogens. Currently, many patients still choose guided tissue regeneration (GTR) with a blocking membrane, recommended for periodontal regenerative therapy in Japan. Thus, synthetic peptides should be developed. To make the best use of clinical data obtained over approximately a decade concerning 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. MALDI-TOF analysis of the eosinophilic corpuscles demonstrated that fragments contained the same amino acid sequence, WYQNMIR. Database analysis demonstrated that the fragments were a swine amelogenin II precursor. A peptide based on the sequence was synthesized. The peptide was subcutaneously inoculated into the backs of rats. Two weeks later, the peptide induced bone, cartilage and endochondral ossification, suggesting hard tissue inducibility. Specifically, the peptide may function as a growth factor to induce cell differentiation in tissue regeneration engineering. The peptide should be investigated both in vitro and in vivo. Optimal concentrations should be determined in vitro and in vivo to investigate the functions of novel growth factors. Various growth factors used for regenerative therapy, such as platelet-rich plasma (PRP), platelet-derived growth factor (PDGF) and basic fibroblast growth factor (b-FGF), have been investigated and clinically applied. For clinical application, optimal concentrations should be determined in vitro and in vivo. Graziani et al. examined the effects of PRP on osteoblasts and fibroblasts, and demonstrated that PRP had dose-dependent effects and that increased doses were not necessarily effective for tissue regeneration. 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 significantly effective at 50 ng / mL. However, the effects remained the same at 50 ng / mL or above. Thus, 50 ng / mL was the optimal dose for treating periodontal wound. Takayama et al. examined the effects of PDGF-BB on human periodontal ligament cells to demonstrate that PDGF-BB had dose-dependent effects on their proliferation and that PDGF-BB had higher ALP activity at lower concentrations during differentiation into calcified tissue. 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 with the sources of cells.

Many reports have been published on Emdogain®, which was used to produce the novel synthetic peptide. Bosshardt summarized reports published over the past 10 years on the responses of various cells to Emdogain®. The responses of the progenitor cells of bone marrow cells or osteoblasts to Emdogain® have most frequently been reported. Differentiation from progenitor cells may lead to tissue regeneration. Hence, the responses of bone marrow cells to the novel synthetic peptide should also be investigated to produce GTR materials.

We herein examined the effects of the novel synthetic peptide on human gingival epithelial cells. Regarding cell proliferation, adhesion and migration, the peptide was effective in suppressing the proliferation of epithelial tissue immediately after periodontal surgery until periodontal regeneration and in facilitating postoperative wound healing. In periodontal regeneration therapy, as understood according to GTR principles, the growth inhibition of epithelial cells and the proliferation of mesenchymal stem cells are required to make space for a regenerative region and strong connective tissue adhesion, instead of epithelial adhesion.

The optimal concentration of the novel synthetic peptide produced on the basis of basic research on EMD remains unknown. We herein used rat bone marrow mesenchymal cells as a model of mesenchymal stem cells to examine their proliferation, adhesion...
and migration and to determine the optimal concentration of the peptide. Then, the effects of the peptide on the potentiality of hard tissue formation at the optimal concentration were examined.

**MATERIAL AND METHODS**

**Cell culture**

RBM cells were isolated from the femurs of 7-week-old Sprague-Dawley rats. This study was performed under the Guidelines for Animal Experimentation at Osaka Dental University (Approval No. 11-04021). Briefly, rats were euthanized using 4% isoflurane, and the bones were aseptically excised from the hindlimbs. The proximal end of the femur and the distal end of the tibia were clipped. A 21-gauge needle (TERMO, Tokyo, Japan) was inserted into the hole in the knee joint of each bone, and the marrow was flushed from the shaft with culture medium (Eagle’s minimal essential medium, EMEM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Life Technologies Corp., CA, USA), penicillin (500 U/mL) (Cambrex Bio Science Walkersville Inc., MD, USA), streptomycin (500 μg/mL) (Cambrex Bio Science Walkersville Inc.) and fungizone (1.25 μg/mL) (Cambrex Bio Science Walkersville Inc.). The resulting marrow pellet was dispersed by trituration, and the cell suspensions from all bones were combined in a centrifuge tube. RBM cells were cultured in 75 cm² culture flasks (Falcon, Becton Dickinson Labwave, NJ, USA) in culture medium. The cells were cultured at 37 °C in a humidified 5% CO₂ / 95% air atmosphere. At confluence, cells were removed from flasks by trypsinization and washed twice in PBS, resuspended with culture medium and seeded. Cells at 3-5 passages were used in the following experiments.

**Peptide synthesis**

The oligopeptide derived from Emdogain®, WYQNMIR, was prepared by traditional solid-phase peptide synthesis in conjunction with the “tea-bag” methodology using Boc/benzyl-based chemistry.

**Cell proliferation assay**

RBM cells were harvested and seeded at a density of 2 × 10⁴ cells/well into 96-well microplates (Falcon, Becton Dickinson Labwave). After 24 hours, the medium was replaced by RBM cells with the synthetic oligopeptide at concentrations of 0, 20, 100, 500 and 1,000 ng/mL. Once the microplates were washed with PBS, cell proliferation/viability was determined using CellTiter-Blue™ Cell Viability Assay Kit (Promega, WI, USA) according to the manufacturer’s instructions, after 1, 3, 6 and 72 hours of incubation. First, we diluted the CellTiter-Blue™ reagent 6 times using PBS. After aspiration of supernatant following the culture and irrigation in PBS, 100 μL of reagents was added to each well, followed by 1 hour of incubation at 37 °C in a humidified 5% CO₂ / 95% air atmosphere. We measured intensity of fluorescence (excitation 560 nm, emission 590 nm) using multimicrotiter readers (SpectraMax® M5, Molecular Device Inc., CA, USA).

**Cell adhesion assay**

Cell adhesion assay was carried out by the method described previously. Synthetic oligopeptide at concentrations of 0, 20, 100, 500 and 1,000 ng/mL was divided into 50 μL samples in 96-well ELISA plates (Nalgene Nunc, Denmark) and incubated at 37°C for 3 hours. Then, the reagent was removed and 1.2% BSA (Sigma, MO, USA) in PBS was divided into 200 μL samples into the same plate for another 1 hour at the same temperature. This plate was used as the test plate.
RBM cells were harvested and seeded at a density of $2 \times 10^4$ cells / well, and then 100 μL of the cell suspension was added to each well of the plates, followed by incubation at 37°C for 1 hour. Subsequently, each sample was shaken for 5 seconds and fixed with 150 μL of 4% paraformaldehyde phosphate buffer (Nacalai Tesque Inc., Kyoto, Japan) in each well. The plate was left for 10 min at room temperature. Then, the attached cells were fixed and stained with 0.25% PicoGreen® dsDNA solution (Invitrogen, Life Technologies Corp., CA, USA) for 5 min. The plates were read in an enzyme-linked immunosorbent assay (ELISA) reader (SpectraMax® M5) at excitation 450 nm and emission 530 nm.

Cell chemotaxis assay
To investigate the chemotaxis activity of RBM cells, a modified Boyden chamber assay was performed using a 24-well microchemotaxis chamber (Fluoroblock insert system; Falcon) as described previously. Equal numbers of RBM cells were suspended and preloaded with the non-toxic fluorescent indicator, 4 μM Calcein™ AM (Molecular Probes, Inc., OR, USA), followed by incubation for 30 min at 37°C. Cells were trypsinized (0.5% trypsin, 5 min), washed in medium, harvested and seeded at a density of $4 \times 10^4$ cells / well into 24-well microplates (Falcon), and then resuspended in culture medium (EMEM, serum-free) (Falcon) as described previously. Next, 750 μL of each medium showing peptide synthesis (0, 20, 100, 500 and 1,000 ng / mL) and medium without synthetic oligopeptide was added to wells in the lower chamber plate by carefully pipetting factor solution between the walls of the upper and lower chambers.

The contents of the upper and lower wells were separated by 3.0 μm pore size HTS FluoroBlock™ Insert (Falcon). Cell chemotaxis was observed for 1, 3 and 8 hours, at 37 °C in a humidified 5% CO₂ / 95% air atmosphere. RBM cells that had passed through the filter to its lower surface were evaluated in a fluorescence plate reader (SpectraMax® M5) with excitation at 485 nm and emission at 535 nm.

Alkaline phosphatase activity
The optimal concentration was determined on the basis of the results of cell proliferation, adhesion and chemotaxis. In the test group, RBM cells were cultured in a suspension with the oligopeptide at optimal concentration and osteogenic supplements (10 mM β-glycerophosphate (Wako), 80 mg / mL ascorbic acid (Nacalai Tesque Inc.) and 10 nM dexamethasone (Nacalai Tesque Inc.). In the control group, they were cultured in a medium without the oligopeptides. After 7 and 14 days of culture, cells were washed with PBS, lysed with 200 μL of 0.2% Triton X-100 (Sigma) and the lysate was transferred to a microcentrifuge tube containing a 5 mm hardened steel ball. Tubes were agitated on a shaker (Mixer Mill Type MM 301, Retsch GmbH & Co., Germany) at 29 Hz for 20 seconds to homogenize the sample. ALP activity was measured using the Alkaline Phosphatase Luminometric ELISA Kit (Sigma) according to the manufacturer’s protocol. The reaction was terminated with 3 N NaOH to a final concentration of 0.5 N NaOH and p-nitrophenol production was measured by absorbance at 405 nm using a 96-well microplate reader (SpectraMax® M5). DNA content was measured using the PicoGreen dsDNA Assay Kit (Invitrogen) according
to the manufacturer’s protocol. To normalize ALP activity, the amount of ALP was normalized to the amount of DNA in the cell lysate.

**Mineralization**
Calcium deposited in the extracellular matrix was measured after dissolution with 10% formic acid. The amount of calcium was quantified using a Calcium E-test Kit (Wako Pure Chemical Industries Ltd.). After 14 days of culture, 1 mL of Calcium E-Test reagent and 2 mL of kit buffer were added to 50 μL of collected medium, and the absorbance of the reaction products was measured at 610 nm using a 96-well microplate reader (SpectraMax® M5). The concentration of calcium ions was calculated from the absorbance value relative to a standard curve.

**Statistical analysis**
Data are presented as mean ± standard deviation, and all data were subjected to one-way analysis of variance (ANOVA).

**RESULTS**

**Effect of Emdogain®-derived synthetic oligopeptide on cell proliferation of RBM**
The effect of the synthetic oligopeptide on cell proliferation of RBM measured by CellTiter-Blue™ Cell Viability Assay is shown in Fig. 1. As Fig. 1 shows, after 1 hour of application, there was no significant difference in the fluorescence intensity between the 100 ng / mL oligopeptide application group and the medium without oligopeptide application group. After 3, 6 and 72 hours of application, the fluorescence intensity in the 100 ng / mL oligopeptide application group was greater than that in the other group. The proliferative response was enhanced by the oligopeptides with exposure time, and remarkable cell proliferation was found with the 100 ng / mL application after 72 hours.

**Effect of Emdogain®-derived synthetic oligopeptide on cell adhesion of RBM**
Fig. 2 shows that cell adhesion in the 100 ng / mL oligopeptide application group was greater than that in the other application group at 1 hour after seeding RBM.

**Effect of Emdogain®-derived synthetic oligopeptide on cell chemotaxis of RBM**
We used CalceinAM for staining of the RBM cells. Because CalceinAM is effective as a fluorescent dye for approximately 8 hours, we performed measurements at 1, 4 and 8 hours after the beginning of RBM cell chemotaxis. The

![Fig. 1](image_url) Fig.1 After 1, 3, 6, and 72 hours of application the fluorescence intensity at the 100 ng / mL oligopeptide application group was greatest of the other application group.
effect of the synthetic oligopeptide on cell chemotaxis of RBM cells measured by a modified Boyden chamber method is shown in Fig. 3. In terms of concluding FBS or not, the cell chemotaxis in the positive control group was significantly greater than that in the negative control group at every application time. After 1, 3 and 8 hours after application, the cell chemotaxis in the 100 ng/mL oligopeptide application group was greater than that in the other application group.

Fig. 2. Cell adhesion at the 100 ng/mL oligopeptide application group was greatest of the other application group at 1 hour after seeding RBM.

Fig. 3. The effect of Emdogain®-derived synthetic oligopeptide on cell chemotaxis of RBM was measured by modified Boyden chamber method. After 1, 3, hours of application the cell chemotaxis at the 100 ng/mL oligopeptide application group was greatest of the other application group.
ALP activity

100 ng/mL oligopeptide was determined as the optimal concentration. Cell differentiation was assessed by measuring the activity of a differentiation marker, ALP, in the test and control groups at 7 and 14 days. At both time points, ALP activity was significantly higher in the cells of the test group compared with the control group (Fig. 4).

Mineralization

Fig. 5 shows calcium deposition in the extracellular matrix of RBM cells in the test and control groups at 14 days. Ca deposition was cumulative in the culture wells, so that measured levels normally increased with exposure time. As shown in the figure, significantly more calcium was deposited by cells in the test group at 14 days than by cells in the control group.

DISCUSSION

We herein examined the effects of an Emdogain®-derived synthetic peptide on the proliferation and differentiation of mesenchymal cells. First, cell kinetics immediately after stimulation, that is, cell proliferation, adhesion and migration, were examined at five concentrations to determine the optimal concentration. Cell differentiation into hard tissue was examined at the optimal concentration. Of the five concentrations, 100 ng/mL peptide stimulation provided the highest proliferation, adhesion and migration capacities. Thus, 100 ng/mL was determined to be the optimal concentration. The 100 ng/mL peptide stimulation significantly increased the ALP activity on days 7 and 14 and intracellular calcium on day 14 after the start of culture in the experimental group compared with that in the control group.

Periodontal growth and regeneration require cementogenesis in intraosseous defects with inflammatory tissue dissected and novel periodontal tissue formation with cells growing to form alveolar bone and periodontal ligament. The most rapidly growing tissue in early wound healing, forming a gingival full-thickness flap, during periodontal surgery is gingival epithelium. Epithelial cells proliferate to facilitate apical growth
deep into the epithelial tissue and form long junctional adhesion between the epithelium and the tooth roots. However, this healing prevents new connective tissue adhesion to preclude periodontal regeneration. During early healing, suppressing deep epithelial proliferation until new periodontal tissue formation occurs is required to achieve regeneration. Regeneration inducers, such as Emdogain®, PRP, and PDGF, which have been recently used, are “growth factors” to stimulate various cells and induce regeneration. The Emdogain®-derived synthetic peptide also functions as a “growth factor.” Early proliferation of mesenchymal cells, that is, periodontal ligament cells or bone marrow mesenchymal cells is required to repair periodontal tissue defects. In particular, immediately after application, an increased number of cells, rather than being induced to differentiate into hard tissue, are required to exhibit suppression of deep epithelial proliferation. Thus, we postulated that cell proliferation; adhesion and migration capacities should be examined to determine the optimal concentration.

Various reports have been published on early cell behavior according to growth factors and cell types. Reports have been published on Emdogain®, from which the synthetic peptide was obtained. Song et al. reported that Emdogain® dose-dependently increased the proliferation of human bone marrow cells over time. They also reported that 200 μg/mL Emdogain® increased cell proliferation and ALP activity to the highest levels, suggesting that 200 μg / mL was the optimal concentration. Guida et al. examined the cell proliferation and ALP activity of 12.5, 25 and 50 μg / mL Emdogain, and demonstrated that Emdogain® dose-dependently increased the cell proliferation and decreased the ALP activity. This suggests that the proliferation of mesenchymal cells and differentiation into osteoblasts are opposite events. However, the synthetic peptide was examined within a narrow concentration range. Thus, an optimal concentration may also exist for the synthetic peptide. No reports have been published on adhesion between bone marrow cells and Emdogain® at several concentrations. Keila et al. reported high early adhesion capacity between Emdogain® and RBM cells compared with that in a control group. We also demonstrated the high adhesion capacity and optimal concentration of the synthetic peptide. In addition, no reports have been published on cell migration using bone marrow cells and Emdogain®. We herein examined the effects of the synthetic peptide on the migration capacity of bone marrow cells by referring to a report on the effects of growth factors on the migration capacity of vascular endothelial cells. In the present study, media with and without FBS in the lower chamber of a filter, into which a cell differentiation inducer was added, served as positive and negative controls, respectively. As shown in Figure 4, compared with the control, the synthetic peptide efficiently facilitated the migration of bone marrow cells at 1 hour after the start of the cell migration. Several reports have been published on the effects of various physiologically active substances, including Emdogain®, on the migration of vascular endothelial cells. Significant difference diminished with migration time. Immediately after application, no significant difference was noted between 100 ng / mL and the other concentrations. The synthetic peptide significantly increased migration capacity compared with the positive control, suggesting that the peptide serves as a material to induce regeneration.

The potentiality of hard tissue formation was examined at the optimal concentration of 100 ng / mL, which was
determined on the basis of results of early cell behavior, using ALP activity and calcium deposition, which indicates calcification. Various reports have been published on the application of Emdogain® to bone marrow cells for hard tissue formation. However, the application remains controversial. Song et al. reported that ALP activity was significantly higher in human bone marrow cells. However, Guida et al. reported that ALP activity, osteocalcin production and calcium deposition were significantly lower in human bone marrow cells. Dolder et al. reported that Emdogain® had no significant effects on the proliferation and differentiation of RBM cells. These inconsistent findings may have resulted from the mechanism of action and cell conditions. In fact, the effects of Emdogain on preosteoblasts and bone marrow cells remain controversial. However, many reports have been published on the significant effects of Emdogain® on hard tissue formation. We herein demonstrated significant differences in ALP activity and calcium deposition. In the present study, cell proliferation was examined at 1-72 hours, ALP activity at 1 week and calcium deposition at 2 weeks. This suggests that the Emdogain®-derived synthetic peptide initially facilitates cell proliferation and subsequently induces differentiation into osteoblasts.

Thus, the Emdogain®-derived synthetic peptide was effective at the optimal concentration in facilitating hard tissue formation during periodontal regeneration in terms of the proliferation, adhesion and migration of RBM cells.

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