Effect of Emdogain®-derived Oligopeptide

in Human Microvascular Endothelial Cells *in vitro*

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Running Title: HMVEC responses to EMD-derived peptide

Abstract

Purpose: Emdogain®(EMD) is derived from the tooth germ of juvenile swine, and is a commonly used for periodontal tissue regeneration, including the formation of alveolar bone, in the treatment of periodontitis. However, because it originates from pig tissue, some patients choose not to be treated with EMD. The active component of EMD is a peptide sequence that corresponds to an amelogenin II precursor. Angiogenesis is one of the most critical events in the wound healing process and in periodontal regeneration. As such, this peptide may function as an angiogenic factor to stimulate cell differentiation and tissue regeneration.

Methods: We characterized the effects of the synthetic peptide derived from EMD on the proliferation, migration, outgrowth extension and ICAM-1 expression in human microvascular endothelial cells (HMVECs).

Results: We demonstrated that the EMD-derived peptide significantly increased HMVEC proliferation and chemotaxis over unstimulated controls. The peptide also led to an increase in outgrowth of processes from HMVEC spheroids in three-dimensional collagen cultures. ICAM-1 mRNA expression was also significantly elevated in HMVECs following treatment with the EMD-derived peptide.

Conclusion: EMD-derived synthetic peptide may act as an angiogenic factor to stimulate the proliferation, chemotaxis, adhesion and migration of microvascular endothelial cells.

Keywords: peptides, enamel matrix derivative, endothelial cells

Introduction

The enamel matrix derivative, Emdogain® (EMD)[1)], has been clinically shown to enhance regeneration in intrabony periodontal defects sites, with an excellent safety profile. Derived from the tooth germ of juvenile swine, this compound has been shown to generate acellular cementum and facilitate periodontal tissue regeneration in patients with periodontitis characterized by marked alveolar bone resorption[2-3)]. EMD has been clinically used for more than a decade to produce marked regenerative effects[4-6)]. In light of these pronounced effects, the molecular mechanisms underlying the action of EMD on the structural cells of periodontal tissue have been comprehensively investigated[7-9)]. In Japan, periodontal bio-regenerative therapy with EMD is being established and has come to be recognized as “advanced medicine”.

Despite its proven efficacy, some patients reject EMD treatment because of concerns about the unknown swine pathogens. Within the literature, there are numerous options for periodontal regenerative therapy based on synthetic materials, such as the use of guided tissue regeneration with resorbable membranes or the use of bone graft materials. Therefore, to capitalize on the already proven efficacy and extensive clinical data surrounding the postoperative stability of tissue generated with EMD, and concomitantly circumvent the current concerns associated with the use of pig sources of materials, there is reason to explore the use of an EMD-derived synthetic peptide.

During the early stages of defining EMD, the compound was subcutaneously injected into the dorsal surface of rats, where it led to the formation of eosinophilic corpuscles and hard tissue including bone and cartilage[10)]. Using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, it was revealed that fragments from these eosinophilic corpuscles contained the amino acid sequence, WYQNMIR,

which was later demonstrated to correspond to the swine amelogenin II precursor sequence using database analyses. A peptide based on the sequence was synthesized, and subcutaneously inoculated into the backs of rats and applied to the periodontal defects of rats. Subsequently[11] , the peptide induced hard tissue such as tissue bone on cementum –like tissue. Specifically[11], it is likely that the peptide functions as a growth factor to induce cell differentiation[12]. Consequently, the activity of the peptide should be investigated both *in vitro* and *in vivo* for its potential utility in bone tissue engineering applications.

EMD has also been extensively investigated across a variety of cell types. For example, Bosshardt *et al.* reviewed changes in the enamel matrix proteins at the cellular and molecular levels as biological mediators for periodontal regeneration[7]. We, too, have previously reported on the responses of gingival epithelial cells, periodontal ligament fibroblasts, periodontal ligament stem cells, and bone marrow mesenchymal cells following the oligopeptide application[13-16]. Given the potential utility of the EMD-derived peptide, we sought to investigate its effects on human microvascular endothelial cells, which are indispensable for periodontal regeneration.

Materials and methods

Cell culture

The commercially available human microvascular endothelial cell line (HMVEC; Gibco; Life Technologies Corporation, Carlsbad, CA, USA) was used in the present study to represent periodontal endothelial cells. HMVECs were cultured in endothelium growth medium, HuMedia-MvG (Kurabou, Osaka, Japan), supplemented with 5% Fetal Bovine Serum (FBS), 10 ng/mL recombinant human epithelial growth factor (rhEGF), 1 μg/mL hydrocortisone hemisuccinate, 50 μg/mL gentamicin, 5 ng/mL amphotericin B, 5 ng/mL recombinant human fibroblastic growth factor-B (rhFGF-2), 10 μg/mL heparin, and 39.3 μg/mL dibutyryl cyclic AMP (dbcAMP) (Kurabou). The cells were cultured in 75 cm^2 culture flasks coated with type I collagen (Asahi Technoglass Inc., Tokyo, Japan) at 37°C in a humidified 5% CO_2/95% air atmosphere. The media was changed every 3 days, and the cells were harvested and seeded until they reached subconfluence. The cells were detached from the flasks by trypsinization (0.5% trypsin, 5 min) and washed twice in PBS, resuspended in culture medium and re-seeded. Cells from passage 4–6 were used in the following experiments.

Synthetic oligopeptide

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

Cell proliferation/viability assay

HMVECs were harvested and seeded at a density of 4×10^3 cells/well into 96-well microplates coated with type I collagen (Asahi Technoglass Inc.). After 24 h, the

medium was replaced by endothelium HuMedia-EB2 basal medium (Kurabou) containing 100 ng/mL of synthetic peptide and 10% FBS. As a control, cells were also supplemented with medium containing 10% FBS without the peptide. The cells were then incubated for 30 min or 1, 3, 6, 24 and 72 h. The microplates were washed with PBS and cell proliferation/viability was determined using CellTiter-Blue™ Cell Viability Assay (Promega, Madison, WI, USA) according to manufacturer's protocol. Briefly, the supernatant was aspirated from the wells, and the cells incubated with 100 µL of CellTiter-Blue™ Reagent diluted 6-fold in PBS for 1 h 37°C. The fluorescence was measured using a multi-microtiter reader (SpectraMax M5, Molecular Device Inc, Sunnyvale, CA, USA) with an excitation of 560 nm and emission of 590 nm.

Cell chemotaxis assay

To investigate HMVEC chemotaxis, a modified Boyden chamber assay was performed using a 24-well microchemotaxis chamber (Fluoroblock Insert system; Falcon, BD Biosciences, Franklin Lakes, NJ), as previously described. Equal numbers of HMVECs were suspended and preloaded with the non-toxic fluorescence indicator Calcein^{AM} (4 µM; Molecular Probes; Life Technologies Corporation, Carlsbad, CA, USA) and incubated for 30 min at 37°C. Cells were trypsinized (0.5% trypsin, 5 min), washed in medium, harvested and seeded at a density of 4×10^4 cells/well in 24-well microplates (Falcon, BD ,Franklin Lakes,NJ, USA) and resuspended in growth medium without FBS to yield a final cell concentration of approximately 2×10^4 cells/500 µL cell suspension per cell culture insert (upper chamber). Next, 750 µL of medium containing 100 ng/mL synthetic peptide, FBS or nothing (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 FluoroBlock™ Insert system (Falcon). Cell chemotaxis was observed for 1, 3, and 8 h at 37°C in a humidified 5% CO_2/95% air atmosphere. HMVECs that passed through the filter into the lower chamber were evaluated on a fluorescence plate reader (SpectraMax® M5; excitation 485 nm, emission 535 nm).

In vitro tube formation assay

This assay is measured using an *In Vitro* Angiogenesis Assay Kit (Cayman Chemical Company,Ann Arbor, MI, USA). Briefly, each well of the chamber slide (Matsunami, Osaka, Japan) was coated with 40 μL of Cell-Based Extracellular Matrix Gel (Cayman Chemical Company), and incubated for 1 min with gentle shaking to ensure an even distribution of the gel. The coated chamber slide was then incubated at 37°C for 60 min to allow the gel to solidify. We then seeded 1×10^5 cells/mL into each gel-coated well in a volume of 100 μL of medium containing 100 ng/mL synthetic peptide or 1mM phorbol-myristate-acetate (PMA) as a positive control. After 6 days of culture, each well was stained with calcein staining solution and the cell networking structure was assessed using an inverted fluorescence microscope (Keyence, Osaka, Japan).

ICAM-1 gene expression levels using Real-time Polymerase Chain Reaction

HMVECs were seeded at an initial density of 4×10^5 cells/well in 24-well type I collagen-coated plates and incubated in 1 mL growth medium containing all supplements (Asahi Technoglass Inc.). After 24 h, the media was replaced with serum-free media (control) or 100 mg/mL EMD dissolved in serum-free media (test). After 3 days of culture, the supernatants were discarded, and the cells were washed with PBS. Total cellular mRNA was extracted from cells using QIAcube® and RNeasy

Mini Kit (Qiagen®; Hilden, Germany) according to the manufacturer's instructions. The mRNA concentration was measured at A260/280. Aliquots of 1 μg total RNA were converted into complementary DNA (cDNA) using a Superscript™ VIRO Synthesis System (Invitrogen; Life Technologies Corporation, Carlsbad, CA). Quantitative real-time PCR was performed using a StepOne Plus™ Real-Time RT-PCR System (Applied Biosystems; Life Technologies Corporation, Foster City, CA, USA). Briefly, using a Fast 96-well Reaction Plate (0.1 mL well volume; Applied Biosystems), 10 μL of Taqman Fast Universal PCR Master Mix, 1 μL of ICAM-1 primer (Taqman Gene Expression Assays), 2 μL of sample cDNA, and 7 μL of DEPC water (Nippongene, Tokyo, Japan) were added to each well. The plate was subjected to 40 reaction cycles of 95°C for 1 s, and 60°C for 20 s. The reactive gene expression rate was calculated by employing the ΔΔCt method in the test group assuming the gene expression rate of the control group. The housekeeping gene GAPDH was used as endogenous control for target gene expression evaluation. Amplification was initially incubated at 50°C for 2 min and then at 95°C for 10 min, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 min.

Statistical analysis

All experiments were performed in triplicate. Data are presented as the mean ± standard deviation, and all data were subjected to Student's *t*-test. In all assays, P-values less than 0.05 were considered significant.

Results

EMD-derived synthetic peptide increases HMVEC proliferation

The effect of the synthetic peptide on the proliferation of HMVECs was measured using a fluorescence-based CellTiter-Blue™ Cell Viability assay and the results are shown in Figure 1. At all of the time points tests (30 min and 1, 3, 6, 24 and 72 h), we found that there was a significant increase in the fluorescence intensity for cells treated with the peptide (peptide group) as compared with cells treated under control conditions (control group) ($P<0.05$).

EMD-derived synthetic peptide increases chemotaxis in HMVECs

HMVECs were stained with Calcein^{AM}. Because the fluorescence lifetime of Calcein^{AM} is approximately 8 h, fluorescence was measured at 1, 3 and 8 h after the commencing the chemotaxis assay. The effect of the synthetic peptide on the chemotaxis of HMVECs was measured using a modified Boyden chamber method. We found that chemotaxis was significantly higher for cells exposed to the peptide as compared with the control cells at all of the time points tested, and that this increase in chemotaxis was similar to that observed for cells exposed to medium containing FBS, which was used in this assay as a positive control ($P<0.05$; Figure 2).

Tube formation after stimulation of EMD-derived synthetic peptide

To further assess the biological activity of the peptide on endothelial cells, we observed its ability to stimulate angiogenesis *in vitro* using a three-dimensional endothelial cell cultivation system. As shown in Figure 3, HMVECs spheroids were stimulated to sprout in a collagen gel in the absence or presence of the peptide, and capillary sprouts grew radially in all three dimensions 6 days after stimulation. In the

absence of the peptide stimulation, only a few short capillary sprouts could be seen originating from the HMVEC spheroids (Fig. 3A,B). The addition of the peptide stimulated capillary sprouting and resulted in an increase in the length and number of the capillary sprouts (Fig. 3C,D), which resembled that observed when the cells were stimulated with PMA (positive control; Fig. 3E,F). These results indicate that the peptide exerts a proangiogenic effect on capillary sprout formation of HMVECs.

EMD-derived synthetic peptide increases ICAM-1 expression in HMVECs

Finally, we tested the effect of the peptide on ICAM-1 gene expression using real-time PCR as a marker of adhesion (Fig. 4). Relative to GAPDH levels, we observed a significant increase in ICAM-1 expression with 50 mg/mL peptide as compared with the control conditions ($P<0.05$).

Discussion

Wound healing and regeneration are a complex processes requiring the interaction between various cells and factors, increased rates of angiogenesis and the initiation of an inflammatory reaction[17]. The process of wound healing is characterized by a series of overlapping stages that include inflammation, granulation tissue formation and tissue remodeling. One of the key elements in granulation tissue formation is angiogenesis or neovascularization[18]. Angiogenesis is a biological mechanism of new capillary formation and requires the activation, migration and proliferation of endothelial cells from pre-existing vessels[19]. Herein, we examined the effects of an EMD-derived synthetic peptide on the cellular kinetics of human microvascular endothelial cells.

Previously, we had determined that 100 ng/mL was the optimal concentration of peptide for *in vitro* assays. Yasui *et al*[16]. suggested that the peptide derived from EMD induces hard tissue formation in Sprague-Dawley rat bone marrow cells, and Kato *et al*[15]. also reported that the peptide in culture with osteogenic medium can significantly enhance the proliferation of periodontal ligament stem cells, as well as the activity of alkaline phosphatase, the expression of osteonectin and osteocalcin, and the formation of calcified nodules in mineralized tissue. Because periodontal tissue regeneration requires angiogenesis in order to regenerate new tissue ingrowth into intraosseous defects, we assumed that this peptide concentration would also optimal for endotheial cells in our experiments.

Our results suggest that the peptide can act as an angiogenic factor in wound repair, as it increased cell proliferation, chemotaxis and the expression of cell adhesion molecule, ICAM-1 in HMVECs. Many reports have described how EMD functions as a growth factor to promote the proliferation of human endothelial cells. Schlueter *et al*[20].

reported an increase in HMVEC proliferation following EMD stimulation, showing it was effective in promoting angiogenesis around periodontal tissues during regenerative therapy. They showed that proliferation was increased 3 days after EMD stimulation, which was administered in media containing FBS. In our study, we examined the effects of the peptide on HMVEC proliferation in EBM-2 media that also contained FBS without growth factor supplementation, because the growth factor supplement contains factors known to stimulate HMVEC proliferation. Our results are in agreement with those reported by Schlueter *et al.* The degree of proliferation of HMVECs is higher than that of human periodontal ligament cells from the viewpoints of blood supply prior to our results which human periodontal ligament cells differentiate into cementoblasts and osteoblasts.

Kasaj *et al*[21]. demonstrated that human umbilical vein cell migration, as well as proliferation, was increased by EMD. Because alimentation is essential within the regenerative area of periodontal tissue, the activity of EMD as a regenerative material is strongly associated with the increased migration of endothelial cells. There are many similar reports demonstrating EMD-induced endothelial cell migration occurring irrespective of the assay type, endothelial cell type, culture conditions, or EMD concentration. In addition to migration, our findings illustrate that the EMD-derived peptide may also act as a chemotactic factor for endothelial cells, as it increased chemotaxis to the same level as FBS, which is known to be potent chemotactic compound.

We employed a collagen gel assay that creates a three-dimensional endothelial cell culture system to assess the potential for the EMD-derived peptide to stimulate vessel outgrowth from HMVECs spheroids. We showed that both the peptide and the PMA positive control induced remarkably higher outgrowth than the unstimulated

control cells as indicated by Fig 3. Yuan *et al*[22]. also showed evidence for new blood vessel outgrowth following EMD treatment in an animal study but none in the negative control group. Although our results were not quantified, our results indicate an angiogenic effect of the peptide similar to that of the PMA positive control.

Our data show that the peptide upregulates the mRNA expression level of adhesion molecule ICAM-1 in HMVECs as a marker of adhesion. We were unable to carry out a cell adhesion assay in this study because the plates were coated the type I collagen, which is known to encourage cell adhesion and would thus confound the results of our study. ICAM-1, also known as CD54, is encoded by the *ICAM1* gene in humans[23,24]. This gene encodes a cell surface glycoprotein typically expressed on endothelial cells and cells of the immune system. ICAM-1 binds to integrins of type CD11a / CD18, or CD11b / CD18 and is also exploited by rhinovirus as a receptor[25]. ICAM-1 is also an intercellular adhesion molecule that is present in low concentrations in the membranes of leukocytes and endothelial cells. Upon cytokine stimulation, the concentrations greatly increase. ICAM-1 can be induced by interleukin-1 (IL-1) and tumor necrosis factor (TNF) and is expressed by the vascular endothelium, macrophages, and lymphocytes. We choose ICAM-1 as a model of adhesion molecule, because the expression of ICAM-1is connected with that of angiopoietin-2 as angiogenic factor[26]. The increase in ICAM-1 expression indicates that the EMD-derived peptide is not only a ligand for endothelial cells but also acts as an angiogenic factor.

Angiogenesis[27] is essential for hard tissue formation during periodontal regenerative therapy. Previous studies suggest that EMD possesses activity similar to that of growth factors[28]. Our previous studies suggest that the effects of EMD supplementation are similar amongst various periodontal cell types[13-16]. Our findings

here suggest EMD-derived synthetic peptide includes an angiogenic-like factor that can stimulate the proliferation, adhesion and migration of HMVECs.

Conclusion

EMD-derived peptide stimulated the proliferation, migration and cytoskeletal extensions of human microvascular endothelial cells, and increased the expression of the cellular adhesion, ICAM-1. Thus, although further *in vivo* experiments are required, the findings presented here indicate that the EMD-derived peptide may offer a useful angiogenic agent to stimulate the activity of endothelial cells at sites of wound repair.

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ヒト微小血管内皮細胞に対する
エムドゲイン由来合成ペプチドの影響

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和　文　抄　録

幼若ブタの歯胚から抽出されたエムドゲイン®（以下，EMDと略す）は，歯槽骨吸収の著しい歯周炎患者の歯周組織再生，とくに無細胞セメント質を誘導し歯周組織の再生を促す材料として現在広く臨床応用されている．しかし，EMDは生物由来材料のため，未知の病原体の問題点を払拭できず患者からの拒否感があるのも事実であり，生物に由来しない合成ペプチドの開発が望まれていることから，EMDの基礎研究から得た成果をもとに新規合成ペプチドを作製した．

今回，硬組織の分化誘導過程における重要な歯周組織構成細胞であるヒト微小血管内皮細胞に対するEMD由来新規合成ペプチドの影響について検討した．

ヒト微小血管内皮細胞として，市販されている皮膚由来のヒト微小血管内皮細胞を実験に使用し，ヒト微小血管内皮細胞に対する新規合成ペプチドの影響としては，細胞の増殖，遊走，管腔の形成および細胞接着分子の発現について検討した.

実験群では合成ペプチドを 100 ng/ml の濃度で培地に溶解させヒト微小血管内皮細胞に応用し，対照群は合成ペプチド無添加とした. 細胞増殖に関しては，30 分，1，3，6，24，72 時間培養後に，細胞遊走に関しては Boyden chamber 法を改良して，1，3，8 時間後にそれぞれを測定した．管腔の形成は三次元培養を施し培養 6 日後に細胞骨格を蛍光染色し観察した. 細胞接着分子の発現は培養 3

日後の ICAM-1 の発現について比較検討を行なった.

細胞増殖は，すべての培養時間で実験群はコントロール群に比べて有意に高かった．細胞遊走は，すべての培養時間でネガティブコントロール群よりも有意に高く，ポジティブコントロール群とほぼ同様の結果となった．管腔の形成について実験群はコントロール群に比べて網目状構造の広がりが大きかった.

ICAM-1 の発現について実験群はコントロール群に比べて有意に高かった.

これらの結果から，EMD 由来の合成ペプチドは歯周組織再生の過程でみられる硬組織の分化誘導過程の栄養供給に関与するヒト微小血管内皮細胞の増殖，遊走，管腔の形成および細胞接着分子の遺伝子発現を促進することが示唆され

た。

Figure Legends

Fig. 1 Increased HMVEC cell proliferation with Emdogain®-derived peptide. The effect of EMD-derived synthetic peptide on the proliferation of HMVECs was measured using the CellTiter-Blue™ Cell Viability assay. Cells were incubated with 100 ng/ml peptide peptide for 30 min or 1, 3, 6, 24, or 72 h (peptide group) as compared with untreated controls (control). The fluorescence intensity for the peptide group was significantly higher than that for the control group at all of the time points measured. * indicates significant difference ($p < 0.05$).

Fig. 2 Chemotaxis increased with Emdogain®-derived peptide. The effect of EMD-derived synthetic peptide on the chemotaxis of HMVECs was measured using a modified Boyden chamber method. Cells were grown in HuMedia-EB2 basal medium (EB2) and tested with the peptide or fetal bovine serum (FBS) as a positive control. After 1, 3, and 8 h, we observed that the peptide generated the same amount of chemotaxis as FBS, and was significantly higher than the control group. * indicates significant difference ($p < 0.05$).

Fig. 3 Increased angiogenesis with Emdogain®-derived peptide. The effect of EMD-derived synthetic peptide on *in vitro* sprouting was measured using a three-dimensional endothelial cell cultivation system (*In Vitro* Angiogenesis Assay Kit). (C,D) In the absence of the peptide stimulation, only a few short capillary sprouts originated from HMVEC spheroids (arrow). The second image on the right (-2) shows a larger magnification of the first (on the left; -1). (A,B) The addition of the peptide stimulated capillary sprouting and resulted in an increase in the length and number of

capillary sprouts, which resembled that observed with PMA stimulation (E,F, positive control).

Fig. 4 Increased ICAM-1 mRNA expression after 72 hours. ICAM-1 mRNA was quantified by real-time RT-PCR following 3 hours incubation with 100 ng/ml peptide (Peptide). After 3 hours, the expression of ICAM-1 mRNA was significantly higher in the peptide group than in the control group (no peptide supplementation). * indicates significant difference ($p < 0.05$).