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Antimicrobial peptide LL37 promotes vascular endothelial growth factor-A expression in human periodontal ligament cells

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Background and Objective: LL37, originally found in the innate immune system, is a robust antimicrobial peptide. LL37 exhibits multiple bio-functions in various cell types, such as migration, cytokine production, apoptosis, and angiogenesis besides its antimicrobial activity Periodontal ligament (PL) cells play a pivotal role in periodontal tissue regeneration. Based on these findings, we hypothesized that LL37 can regulate PL cell function to promote regeneration of periodontal tissue. To prove this hypothesis, we investigated the effect of LL37 on the potent angiogenic inducer vascular endothelial growth factor (VEGF) expression in cultures of human PL (HPL) cells because neovascularization is indispensable for the progress of tissue regeneration. Moreover, we investigated the signaling cascade associated with LL37-induced VEGF expression.

Material and Method: HPL cells were treated with synthesized LL37 in the presence or absence of PD98059, a MEK-ERK inhibitor, or PDTC, an NF-κB inhibitor. VEGF expression levels were assessed by real-time polymerase chain reaction analysis and an enzyme-linked immunoassay. Phosphorylation levels of ERK1/2 or NF-κB p65 were determined by Western blotting.

Results: LL37 upregulated VEGF-A expression at the mRNA and protein levels in HPL cells, while VEGF-B mRNA expression was not affected. Both ERK and NF-κB inhibitors clearly abrogated the increase in VEGF-A levels induced by LL37 in HPL cells. Importantly, LL37 increased phosphorylated levels of ERK1/2 and NF-κB p65 in HPL cells.

Conclusion: LL37 induces VEGF-A production in HPL cells via ERK and NF-κB signaling cascades, which may result in angiogenesis, thereby contributing to periodontal regeneration.

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LL37, the only human member of the cathelicidin family, is formed from the last 37 amino acid residues of the C-terminus of human cationic antimicrobial peptide 18 (1, 2). LL37 is widely expressed in a variety of bodily fluids and tissues, including innate immune cells, such as neutrophils and epithelial cells (1, 3). LL37 possesses a broad-spectrum antimicrobial activity against gram-negative and -positive bacteria, fungi, and viruses (1, 4–6).

Aside from its antimicrobial properties, LL37 also exhibits multiple bio-functions in various cell types, such as cell migration, cytokine production, cell apoptosis, and angiogenesis (7–11).

Promotion of neovascularization during periodontal tissue regeneration is required for its successful regeneration because the newly formed vessel can supply oxygen, nutrition, and progenitor cells to the damaged region thereby triggering the regenerative process. Indeed, some growth factors, such as brain-derived neurotrophic factor and fibroblast growth factor-2, have been widely accepted to induce angiogenesis in periodontal tissue, which in turn facilitates regeneration (12–16). These facts implied that other cytokines or peptides, which can mediate angiogenesis, including LL37, are potentially worthy of study for their application to the regeneration of periodontal tissue defects.

The success of cytokine-mediated angiogenesis (growth of new capillary blood vessels from pre-existing vascular endothelial cells), depends on two types of biological mechanisms, direct and indirect action. Some kinds of cytokines can directly act on endothelial cells to induce their proliferation and neovascularization. As an indirect mechanism, vascular endothelial growth factor (VEGF) is a potent mitogenic growth factor for endothelial cells (17) released from endothelial cells or neighboring cells, such as fibroblasts in an autocrine and paracrine fashion, that initiates endothelial cell proliferation and blood vessel formation. Although LL37 has been reported to directly upregulate the proliferation and formation of vessel like structures on endothelial cells (10), it is still not clear if LL37 accelerates VEGF expression as an indirect mechanism in the process of angiogenesis.

Periodontal ligament (PL) cells consist of heterogeneous cell populations, such as osteoblasts, cementoblasts, or stem cells, and are thought to be a prerequisite for periodontal tissue regeneration. In addition, previous studies have shown that PL cells provide a large variety of cytokines, including VEGF, to maintain periodontal tissue homeostasis (18–20). In other words, it is conceivable that PL cells can be a valuable cellular source of cytokines to accelerate progress of periodontal tissue regeneration.

Accordingly, based on accumulating lines of evidence, we hypothesized that LL37 can regulate VEGF production in PL cells to activate angiogenesis, thereby contributing to the regeneration of periodontal tissue. In this study, to test this tentative concept, we examined the effect of LL37 on VEGF expression in human PL (HPL) cells and investigated the molecular mechanisms underlying this bio-function.

Materials and methods

Synthesis of LL37

LL37 was synthesized using a peptide synthesizer (Shimazu, Tokyo, Japan) as described previously (21). The peptide was purified using reverse-phase HPLC with an octadecyl-4PW column (Tosoh, Tokyo, Japan) and a linear gradient of aqueous 0.05% trifluoroacetic acid to 100% acetonitrile containing 0.05% trifluoroacetic acid, and the sample was then lyophilized to remove the organic solvent. To confirm the purity and quality of the peptide, mass spectrometry, using the matrix-assisted laser desorption-ionization/time-of-flight-mass spectrometry (TOF/MS) method, was performed with a Voyager apparatus (PerSeptive Biosystems, Framingham, MA, USA). TOF/MS analysis revealed that the mass of LL37 was 4174.1 Da, which is identical to that calculated from the primary sequence. Moreover, the obtained spectrum showed a single peak.

Cell Culture

HPL cells were purchased from Lonza (Lonza Walkersville Inc, Basel, Switzerland), cryopreserved at the third passage. Cells were maintained in Dulbecco's modified Eagle's medium (Sigma, Steinheim, Germany) supplemented with 10% fetal bovine serum (Biowest, Nuaillé, France), 100 U/mL penicillin (Sigma), 100 μg/mL streptomycin (Sigma), and 500 ng/mL amphotericin B (Invitrogen, Carlsbad, CA, USA) (medium A). To obtain enough cell numbers for the series of experiments, cells were propagated to the ninth passage. Our preliminary study showed that cell growth and calcification activity was not different between cells at the ninth passage and earlier one. Accordingly, HPL cells at the ninth passage were used in the following experiments. Human microvascular endothelial cells (HMVECs) were a primary cell culture line purchased from Cambrex Bio Science Walkersville (Walkersville, MD, USA), cryopreserved at the third passage. HMVECs were maintained in endothelial basal medium (EBM)-2 purchased from Lonza Walkersville Inc. supplemented with fetal bovine serum, fibroblast growth factor-2, epidermal endothelial growth factor, hydrocortisone, insulin-like growth factor, ascorbic acid, VEGF, and amphotericin B (medium B). HMVECs at passage 5 were used for the following experiments.

Expression of vascular endothelial growth factor mRNA

HPL cells were seeded at a density of 1.0×10^5 cells per well in six-well plastic culture plates and maintained in medium A. After 7 d, HPL cells were pretreated with or without a MEK-ERK inhibitor, PD98059 (10 μM, Merck KGaA, Darmstadt, Germany), a p38 inhibitor, SB203580 (10 μM, Merck KGaA), a JNK inhibitor, SP600125 (10 μM, Merck KGaA), or an NF-κB inhibitor, PDTC (10 μM, Merck KGaA), for 30 min in DMEM supplement with 100 U/mL penicillin, 100 μg/mL streptomycin, and 500 ng/mL amphotericin B (medium C). Subsequently, cells were exposed to increasing concentrations of LL37 (0–10 μg/mL) for the indicated times (0–24 h). HMVECs were seeded at a density of 5.0×10^4 cells per well in six-well plastic culture plate coated with type I collagen and maintained in medium B. After the

confluence, HMVECs were exposed to LL37 (10 μg/mL) for the indicated times (0–24 h) in EBM-2. Total RNA was extracted from cells with RNAiso (Takara, Otsu, Japan) and quantified by spectrometry at 260 and 280 nm. First strand cDNA was synthesized with 2.5 μg of total RNA extract in a total volume of 20 μL using ReverTraAce (Toyobo, Osaka, Japan). The mRNA expressions of VEGF-A, VEGF-B, and GAPDH were quantified by real-time polymerase chain reactions (PCR). GAPDH was used as an internal control. PCR was carried out using a TaqMan® Gene Expression assay (Applied Biosystems, Foster City, CA). Assay ID for VEGF-A, VEGF-B, and GAPDH was Hs00900055_m1, Hs00173634_m1, and Hs02758991_g1, respectively.

Measurement of vascular endothelial growth factor-A levels

HPL cells were seeded at a density of 1×10^4 cells/well in 48-well plastic tissue culture dishes, and maintained in 0.2 mL of medium A. After 7 d, cells were washed three times with DMEM. HPL cells in confluent cultures were then exposed to various concentrations of LL37 in medium C for 24 h before the end of incubation on day 7. The culture supernatants were collected and the protein concentration was measured by Bradford assay kit (Bio-Rad, Hercules, CA, USA). Noteworthy, the total protein concentration in each sample was not significantly different (data not shown). Then, VEGF-A levels in the supernatant were determined using a Human VEGF ELISA Development Kit (Pepro Tech, Rocky Hill, NJ, USA).

Immunoblotting of ERK1/2, NF-κB p65, and p38

HPL cells were seeded at a density of 1.0×10^5 cells per well in six-well plastic culture plates and maintained in medium A. Cells were pretreated with or without PD98059 (10 μM) and PDTC (10 μM) for 30 min and then exposed to LL37 (10 μg/mL) for 20 min before the end of incubation in medium C. HPL cells were washed three times with phosphate-buffered saline (PBS; pH 7.5) and lysed in 250 μL of sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris–HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mM dithiothreitol, and 0.01% w/v bromophenol blue). The cell lysate was sonicated for 5 s at 4°C. Samples were resolved by 10% SDS-polyacrylamide gel electrophoresis under nonreducing conditions and electrophoretically transferred on to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked with 5% skim milk for 1 h and then reacted with rabbit anti-human phospho-ERK1/2 (Cell Signaling, Danvers, MA, USA, 1 : 1000), rabbit anti-human total ERK1/2 (Cell Signaling, 1 : 1000), rabbit anti-human phospho-NF-κB p65 (Cell Signaling, 1 : 500), rabbit anti-human total NF-κB p65 (Cell Signaling, 1 : 500), rabbit anti-human total p38 (Cell Signaling, 1 : 1000), rabbit anti-human phospho-p38 (Cell Signaling, 1 : 1000), or rabbit anti-β-actin (Sigma, 1 : 200). After the membrane was washed, it was incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (R&D Systems, Minneapolis, MN, USA) in Tris-buffered saline [TBS; 20 mM Tris–HCl, 0.5 M NaCl (pH 7.6)] for 1 h at room temperature. After further washing, immunodetection was performed using ECL Plus Western blotting detection reagents (GE Healthcare, Little Chalfont, UK). The density of the bands was analyzed with ImageJ, a java-based image processing software (NIH, Bethesda, MD, USA).

Statistical analysis

The data were analyzed by ANOVA or Student's *t*-test. In a time or dose–course study, differences among groups were analyzed by one-way ANOVA (Fig. 1A–H). Alternatively, differences between groups in an inhibition assay were analyzed with a Student's *t*-test (two-sided) (Figs 2A, B and 3E, F). $p < 0.05$ values were considered significant.

Results

LL37 upregulates vascular endothelial growth factor-A expression in human periodontal ligament cells

mRNA expression of VEGF-A and VEGF-B was examined by real-time PCR, because, among the VEGF family, these two play a crucial role in angiogenesis (22). LL37 promoted VEGF-A mRNA expression, peaking at 3 h and then gradually decreasing (Fig. 1A), and its elevation was in a dose-dependent manner (Fig. 1B). On the other hand, the level of VEGF-B mRNA expression was not affected by LL37 in HPL cells (Fig. 1D and 1E). Intriguingly, LL37 did not stimulate either VEGF-A or VEGF-B mRNA expression in HMVECs (Fig. 1C and 1F). Furthermore, in accordance with the real-time PCR study, LL37 also elevated VEGF-A protein levels in cell culture supernatant from HPL cells in a time and dose-dependent fashion (Fig. 1G and 1H).

Both ERK and NF-κB specific inhibitors abrogated LL37-induced vascular endothelial growth factor-A expression in human periodontal ligament cells

To explore the possible signaling cascade engaged in LL37-induced VEGF-A expression, we performed an inhibition assay. Pretreatment with PD98059 and PDTC suppressed VEGF-A mRNA expression levels induced by LL37 (Fig. 2A), whereas neither SP600125 nor SB203580 attenuated the elevation (data not shown). Furthermore, consistent with mRNA expression levels, both PD98059 and PDTC hampered LL37-induced VEGF-A production (Fig. 2B). These results suggested that LL37 promotes VEGF-A expression via the ERK and NF-κB signaling cascades.

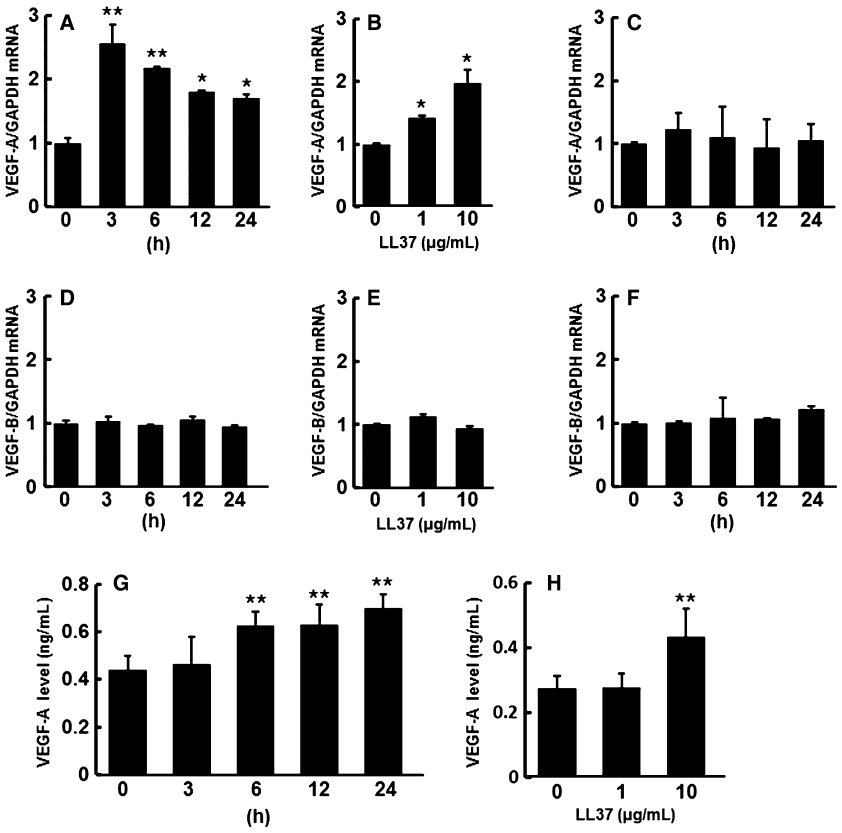

Fig. 1. LL37 upregulates vascular endothelial growth factor (VEGF)-A expression in human periodontal ligament (HPL) cells. (A, C, D, F and G) Time-course effect. HPL cells (A, D and G) or human microvascular endothelial cells (C and F) were exposed to LL37 (10 μg/mL) for the indicated times before the end of incubation on day 7. (B, E and H) Dose-dependent effect. HPL cells were exposed to increasing concentrations of LL37 for 3 h (B and E) or 24 h (H) before the end of incubation on day 7. mRNA expression of VEGF-A (A, B and C) and VEGF-B (D, E and F) was determined by real-time PCR. Graphs show the ratio of VEGF-A or VEGF-B mRNA to GAPDH mRNA (A–F). VEGF-A protein levels were monitored by ELISA (G and H). Three independent experiments were performed and similar results were obtained. Values are mean (± SD) of three cultures in one experiment. $*p < 0.05$, $**p < 0.01$: differs significantly from the control (ANOVA).

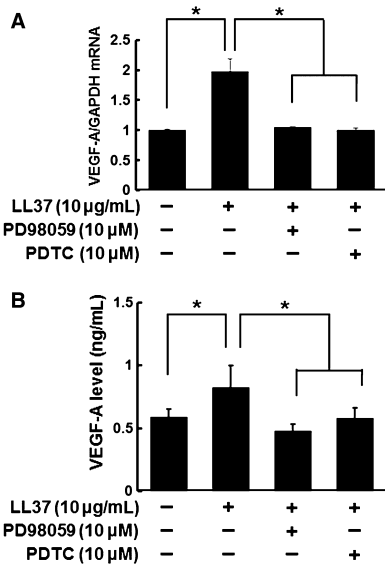

Fig. 2. Involvement of the ERK and NF-κB pathway in LL37-induced vascular endothelial growth factor (VEGF)-A increases in human periodontal ligament (HPL) cells. HPL cells, having been pretreated with or without a MEK-ERK inhibitor (PD98059, 10 μM) or an NF-κB inhibitor (PDTC, 10 μM) for 30 min, were exposed to 10 μg/mL LL37 for 3 h (A) or 24 h (B). (A) mRNA expression of VEGF-A was determined by real-time PCR. Graphs show the ratio of VEGF-A mRNA to GAPDH mRNA. (B) VEGF-A protein levels in culture supernatant were measured by ELISA. Three independent experiments were performed and similar results were obtained. Values are mean (± SD) of three cultures in one experiment. $*p < 0.05$, $**p < 0.01$: differs significantly from the control (*t*-test).

LL37 phosphorylates ERK1/2 and NF-κB p65 in human periodontal ligament cells

LL37 increased phosphorylated ERK 1/2 and NF-κB p65 levels in HPL cells, and the maximal effect was seen at 20 min (Fig. 3A and 3B). The increase in phosphorylated levels of ERK1/2 and NF-κB p65 induced by LL37 was apparently abrogated by pretreatment with PD98059 (Fig. 3C and 3E) and PDTC (Fig. 3D and 3F), respectively. It is noteworthy that PD98059, which obviously attenuated ERK1/2 phosphorylation, did affect neither levels of phosphorylated NF-κB p65 (Fig. 3D and 3F) nor phosphorylated p38 (Fig. S1) increased by LL37. The NF-κB p65 inhibitor, PDTC, failed to diminish the level of phosphorylated ERK1/2 (Fig. 3C and 3E). These findings indicated that LL37 activates the ERK and NF-κB signaling cascades independently in HPL cells.

Discussion

The present study demonstrated, for the first time, that LL37 can evoke the production of VEGF-A from HPL cells, via activation of ERK and NF-κB signaling cascades. Indeed, as ERK and NF-κB signaling cascades are well known to be associated with the production of a raft of cytokines, contributing to various cellular responses (23, 24), the novel finding in this study will give new insight into the application of LL37, which can facilitate such critical signaling pathways to mediate the cytokine expression pattern for tissue regenerative therapy.

Many scientific efforts revealed that LL37, originally found as an antimicrobial peptide in the innate immune system, is engaged in tissue repair or wound healing by regulating epithelial cell proliferation or

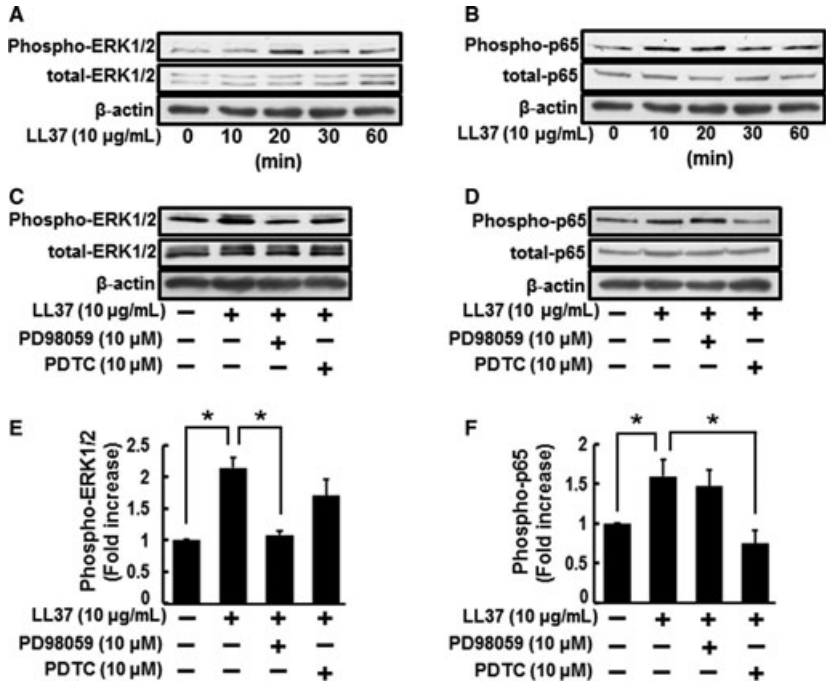

Fig. 3. LL37 increases phosphorylated levels of ERK1/2 and NF-κB p65 in human periodontal ligament (HPL) cells. (A,B) The effect of LL37 on phosphorylation of ERK1/2 (A) and NF-κB p65 (B). HPL cells were exposed to LL37 (10 μg/mL) for the indicated times before the end of incubation on day 7. (C–F) The effect of a MEK-ERK inhibitor and an NF-κB inhibitor on LL37-induced phosphorylated ERK1/2 (C and E) and NF-κB p65 (D,E) levels. HPL cells, having been pretreated with or without a MEK-ERK inhibitor (PD98059, 10 μM), and an NF-κB inhibitor (PDTC, 10 μM) for 30 min, were exposed to 10 μg/mL LL37 for 20 min. Phosphorylated (phospho-) ERK1/2, total ERK1/2, phospho-NF-κB p65 (phospho-p65), total NF-κB p65 (total-p65), and β-actin were determined by Western blotting. The bands are representative of three independent experiments. The graph indicates the density of the bands of phospho-ERK1/2 (E) or phospho-p65 (F). The band density of blots from three independent experiments was measured using an image densitometer. Values are mean (± SD) of three independent experiments. $*p < 0.01$: differs significantly from the control (*t*-test).

migration (7, 25). In addition, Koczulla *et al.* demonstrated that LL37 could also induce angiogenesis in a rabbit hind-limb ischemia model (10). In their study, LL37 elicited endothelial cell proliferation, which contributed to the formation of vessel-like structures. However, it is noteworthy that the neutralizing anti-VEGF antibody did not attenuate the LL37-induced proliferation of endothelial cells, suggesting that an indirect effect of LL37 through the production of VEGF is unlikely. Moreover, consistent with their findings, the present study also showed that LL37 had no effects on VEGF-A or -B mRNA expression in HMVECs. Nonetheless, whether LL37 can induce VEGF production in the process of angiogenesis as an indirect mechanism still needs to be elucidated, because VEGF should be abundantly provided by not only endothelial cells, but also cells neighboring the site of neovascularization, including epithelial cells or fibroblasts (17, 26). In fact, our results apparently indicate that LL37 can elicit VEGF-A production from HPL cells (Fig. 1), which plays a crucial role in the regulation of periodontal tissue homeostasis (27–29). Consequently, in periodontal tissue, besides the direct action by LL37 on endothelial cells, LL37 may regulate angiogenesis by an indirect action via VEGF released from PL cells; however, an additional study will be required to identify the molecular mechanisms of LL37-induced direct and indirect angiogenesis.

It is well demonstrated that LL37 regulates cellular function, such as migration, proliferation, and cytokine production. These series of LL37 studies revealed that LL37 facilitates its bio-effect in the range from 1 to 20 μg/mL or from 0.1 to 10 μM (7, 8, 10, 11, 30). In this study, we tested 10 μg/mL of LL37 for the regulation of VEGF-A production and this concentration is equivalent to 2.4 μM. Therefore, the LL37 concentration tested in this study seems to have a good agreement with previous reports.

LL37 stimulated HPL cells to produce more than 500 pg/mL of VEGF-A in culture supernatant (Fig. 1G and 1H). In the present study, we have not yet confirmed the capability of VEGF-A released from HPL cells to induce angiogenesis. However, as a previous study has reported that the conditioned medium containing about 500 pg/mL VEGF-A can facilitate the migration of endothelial cells (31), it seems feasible that LL37-induced VEGF-A from HPL cells also acts on endothelial cell function to induce angiogenesis.

Cytokine-based therapy for periodontal tissue regeneration has captured broad dental researchers' attention for some time because of its powerful ability to regulate cellular function (12–16, 32–34). A periodontal tissue defect is triggered by an infection of periodontopathogenic bacteria (35), and even after periodontal basic therapy, a patient's oral cavity can harbor numerous microbes, which may impair the cellular function responsible for successful periodontal tissue regeneration. Therefore, a promising remedy for the regeneration of periodontal tissue defects is required with the ability to conquer pathogenic microbial activity as well as regulate cellular function in the regenerative process. As we have previously reported that LL37 exerts an antimicrobial activity against periodontopathogenic and cariogenic bacteria (36), as well as multiple bio-

functions, such as regulating cell migration (7, 8, 11), proliferation (10, 30), or cytokine production, as shown in this study, it is plausible that the application of LL37 may be more effective for periodontal tissue regeneration.

The ERK signaling pathway is one of the major intracellular signalings for various cellular responses, especially cytokine production (23). Regarding the regulation of VEGF expression, fibroblast growth factor-2 accelerates VEGF expression in osteoblasts through the ERK signaling cascade (37). This was supported by Milanini *et al*. (26) who showed that activation of Raf-1 (an upstream regulator of the MEK/ERK pathway) could promote the transcriptional activation of the VEGF gene and elevate VEGF production in hamster fibroblasts. Consistent with these findings, in our present study, a MEK-ERK inhibitor, PD98059, abrogated the elevation of VEGF-A caused by LL37 in HPL cells, whose morphology is fibroblastic, suggesting that the ERK signaling pathway plays a crucial role in the upregulation of VEGF expression in fibroblasts.

The transcriptional factor NF-κB is well known to regulate the expression of multiple genes involved in a wide variety of cellular responses (24). Activation of NF-κB in endothelial cells balances angiogenic factors against angiostatic factors to modulate angiogenesis (38, 39). Furthermore, inhibition of NF-κB results in the suppression of VEGF-A expression on infantile hemangioma-derived stem cells (40). The present study also demonstrated that the obstruction of NF-κB activation significantly attenuated LL37-induced VEGF-A expression in HPL cells. Therefore, activation of NF-κB, as well as ERK signaling, by LL37 in PL cells may be essential to regulate VEGF expression thereby facilitating angiogenesis in periodontal tissue.

In summary, LL37 stimulates ERK and NF-κB activity to induce VEGF expression in HPL cells. Consequently, the culmination of these events may facilitate angiogenesis, thereby contributing to periodontal tissue regeneration. This study shed light on the potential role of LL37, leading to the development of a novel therapeutic remedy for periodontal disease based on its bio-function to regulate VEGF expression, in addition to its antimicrobial activity.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 The effect of a MEK-ERK inhibitor on LL37-induced phosphorylated p38 level. HPL cells, having been pretreated with or without a MEK-ERK inhibitor (PD98059, 10 μM) for 30 min, were exposed to 10 μg/mL LL37 for 20 min. Phosphorylated (phospho-) p38, total p38, and β-actin were determined by Western blotting.

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