# An *In Vitro* Study on the Usefulness of LL37 as a Pulp Capping Material

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**Ph.D. Thesis**

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by

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# Contents

# Chapter One

## Preface

The pulp-dentin complex serves as a physiological barrier of the tooth and protects it against occlusal overload. A continuous outward flow of dentinal fluid prevents the inward diffusion of noxious agents, such as bacteria or their components (1). In addition, throughout the life of a tooth, odontoblasts keep forming peri-tubular and tertiary dentins in respond to biological and pathological stimuli, and these dentins further help to reduce the risk of those noxious agents penetrating into the pulp. The odontoblasts themselves may also release antimicrobial peptides, which can directly kill bacteria (2). Regarding to the protection against occlusal overload, some of the nerves residing in the pulp tissue are thought to have proprioceptor functions. This speculation is supported by a study using cantilever weight to load on vital or non-vital teeth, which demonstrated that non-vital teeth are more prone to fractures because much more weight could be placed on them before pain was experienced (3). Furthermore, the survival rate of root-filled teeth has been reported to be lower compared to that of vital teeth (4). Therefore, maintaining pulp vitality is crucial to the tooth's long-term survival.

Pulp capping is the treatment of an exposed or nearly exposed pulp with a dental material in an attempt to facilitate the formation of reparative dentin and preserve pulp vitality (5). From clinical and economical viewpoints, pulp capping is technically simple to perform and usually can be done in a single visit at a low cost, making it a beneficial procedure for both dentists and patients. However, this type of treatment has its limitations. While it has a high success rate of 92% with mechanically exposed pulps, this is not the case with those exposed by caries, with the

success rate of only 33% (6). The penetration of bacteria to the pulp will result in pulpal inflammation, leaving the pulp less able to respond and heal, compared to a mechanical exposure without preexisting inflammation (7). To improve these clinical outcomes of pulp capping on caries-associated cases, there is a critical need to develop a biological active material, which ideally should have the following properties: bacterial elimination, inflammatory regulation (e.g. pulp tissue breakdown inhibition), pulp cell activation (e.g. migration, proliferation and differentiation into odontoblasts) and angiogenesis stimulation.

Cathelicidins are a family of antimicrobial peptides characterized by a fairly conserved N-terminal prosequence cathelin domain and a variable C-terminal antimicrobial domain. To date, only one member of the cathelicidin family has been identified in human; the unprocessed form is termed human cationic antimicrobial peptide 18 (hCAP18) and the mature form is termed LL37 (8). As the name implies, LL37 is formed by a proteolytic cleavage from the last 37 amino acid residues of the C-terminus of hCAP18, with the first two residues of its sequence being leucine (9, 10). LL37 is expressed in various types of leukocytes and epithelial cells, and is also present in mucosal secretions, sweat and plasma (11-15). This peptide plays a major role in innate immune defense against bacteria, fungi and viruses (16). Its broad spectrum of bactericidal activity owes to its net-positive charge and the propensity to fold into an amphipathic $\alpha$-helix upon contact with bacterial membrane, allowing it to interact with and disrupt the membrane (10). Apart from its main antimicrobial role, LL37 also exhibits a wide range of other biofunctions, including lipopolysaccharide (LPS)/lipoteichoic acid (LTA)-neutralizing activity, chemoattractant function, immunomodulation, the stimulation of angiogenesis and wound healing, and the

mediation of cytokine release (17). In other words, LL37 is a robust molecule capable of inducing diverse biological effects.

Previous studies have demonstrated that LL37 is effective against cariogenic bacteria (18) and promotes pulp cell migration (19). Thus, with these properties, LL37 may be a potential pulp capping material. However, before it can be used for this purpose, LL37 still needs to meet some remaining criteria, which should be whether LL37 has some angiogenic roles in dental pulp and can inhibit/reduce pulp tissue breakdown.

This study aimed to investigate the possible application of LL37 as a pulp capping material by addressing the two remaining criteria, and was accordingly divided into two parts: (1) the angiogenic effect of LL37 on pulp cells and (2) the inhibition/reduction of pulp tissue breakdown by LL37.

# Chapter Two

# The Angiogenic Effect of LL37 on Pulp Cells

## 1. Introduction

Angiogenesis is indispensable for pulp wound healing. The newly formed blood vessels supply nutrients, oxygen, signaling molecules and progenitor cells to the wound site, while simultaneously removing metabolic waste products from the area (20, 21). The endothelial cells lining sprouting capillaries also play a role in pulp homeostasis (21), and may promote the survival of adjacent cells during angiogenesis (22).

Following an injury, angiogenesis is immediately initiated in response to various angiogenic factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF/FGF-2), which are released from the wounded tissue and platelets (23, 24). VEGF (or VEGF-A) is the founding member of the human VEGF family, which consists of other four members: VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF). VEGF itself consists of multiple isoforms, with VEGF-165 being the predominant one followed by the 189 and 121 residue molecules (25). VEGF was originally discovered as a tumor-secreted protein that increases the permeability of microvessels. Therefore, it is also referred to as vascular permeability factor (VPF) (26). However, it was later found to stimulate the proliferation and migration of endothelial cells and induce angiogenesis *in vivo* (27, 28). VEGF is not only highly expressed in tumors, but also in other pathological and physiological conditions characterized by angiogenesis, such as psoriasis, rheumatoid arthritis and ovarian corpus luteum formation (29), and in various normal human tissues and organs (30, 31). VEGF is widely regarded as the single most important

angiogenic factor (32), and its expression, which is upregulated by many factors ranging from hypoxia conditions to certain growth factors and hormones (33-35), is often associated with angiogenesis.

The dental pulp is a highly vascularized tissue and consists mainly of pulp fibroblasts. In addition to their involvement in reparative dentin formation, these pulp cells are known to express VEGF and play a role in angiogenesis (36-38). Therefore, an increase in the release of VEGF from pulp cells may promote the process of pulp wound healing by inducing angiogenesis.

LL37 has been reported to directly activate endothelial cells to increase their proliferation and the formation of vessel-like structures (39). However, since LL37 can also mediate cytokine release from various cell types (40), it remains to elucidate if this peptide is indirectly involved in angiogenesis by inducing the secretion of angiogenic factors from resident cells. To address this issue, the effects of LL37 on the expression of VEGF in human pulp (HP) cells *in vitro* and the intracellular signaling pathway involved in this process were examined.

## 2. Materials and Methods

### *2.1. Cell and Culture Condition*

Three healthy premolars extracted for orthodontic reasons were donated by three different patients with their informed consent according to a protocol approved by the Ethical Authorities at Hiroshima University (D47-2). Pulp cells from each patient were then separately obtained from the explant cultures of pulps removed from their corresponding tooth as previously described (41), and were named HP1, HP2 and HP3 cells. HP cells at passage 6 were used in all the experiments conducted in

this study. Cells were cultured in Dulbecco's modified Eagle's Medium (DMEM; Sigma, Steinheim, Germany) supplemented with 10% fetal bovine serum (Serum Source International Inc., Charlotte, NC), 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO), 100 μg/ml streptomycin (Sigma-Aldrich) and 1 μg/ml fungizone (Invitrogen Life Technologies, Carlsbad, CA) at 37$^{o}$C in a humidified atmosphere of 5% of $CO_2$, with the media being changed 1 day after the seeding and every 3 days afterward. When cells reached confluence after 7 days of culture, they were washed twice with phosphate buffered saline (PBS). Culture media were then changed to serum-free DMEM, and stimulations were started according to each experiment as follows.

*2.2. RNA Preparation and Real-time PCR*

Confluent HP cells, which had been cultured at a density of $1 \times 10^5$ cells/well in six-well plastic culture plates (Corning Inc., Corning, NY) with each well containing 2 ml of medium, were stimulated with 10 μg/ml LL37, synthesized as described previously (42) and kindly provided by Prof. Hitoshi Komatsuzawa of Kagoshima University Graduate School of Medical and Dental Science, for various lengths of time (0-24 h) before the end of the incubation on day 8. For the dose-response assay, cells were treated with LL37 (0-10 μg/ml) for 3 h. To determine the signaling pathway involved, an inhibition assay was conducted by either pretreating cells or not for 30 min with 10 μM PDTC (an NF-κB inhibitor; Merck KGaA, Darmstadt, Germany), 10 μM SB203580 (a p38 MAPK inhibitor; Calbiochem, La Jolla, CA), 50 μM PD98059 (an ERK kinase inhibitor, Calbiochem), or 10 μM SP600125 (a JNK inhibitor, Calbiochem) before being further stimulated with 10 μg/ml LL37 for another 3 h. Total RNA was isolated using RNAiso (Takara, Otsu,

Japan), purified, and quantified by spectrometry at 260 nm and 280 nm. Then, 2.5 μg of the total RNA was reverse transcribed with ReverTraAce (Toyobo, Osaka, Japan), following the manufacturer's protocol. GAPDH was used as a housekeeping gene, and the mRNA expression levels of VEGF were relatively quantified by real-time PCR using comparative CT method. PCR was performed using a TaqMan® Gene Expression Assay (Applied Biosystems, Foster City, CA), with the assay ID for VEGF and GAPDH being Hs00900055_ml and Hs02758991_gl, respectively.

*2.3. Enzyme-linked Immunosorbent Assay (ELISA)*

HP2 cells were chosen as the representative of the three HP cells, and were cultured in triplicate at a density of $1 \times 10^4$ cells/well in 48-well plates, with each well containing 200 μl of medium. After becoming confluent, cells were stimulated with 10 μg/ml LL37 for various lengths of time (0-24 h) before the end of the incubation on day 8 in the time-response assay, or with LL37 (0-10 μg/ml) for 24 h in the dose-response assay. For the inhibition experiment, cells were either pretreated or not for 30 min with 50 μM PD98059 before being exposed to 10 μg/ml LL37 for another 24 h. The supernatants were collected and VEGF protein levels were determined using a Human VEGF ELISA Development Kit (PeproTech, Rocky Hill, NJ), according to the manufacturer's instructions.

*2.4. Immunoblotting*

Representative HP2 cells were seeded at a density of $1 \times 10^5$ cells/well in six-well plates, with each well containing 2 ml of medium, until they reached confluence after 7 days of culture. Cells were then treated with 10 μg/ml LL37 for 10, 20, 30, and 60 min (time-response assay) before the end of the incubation, or were either

pretreated or not for 30 min with 50 μM PD98059 before being exposed to 10 μg/ml LL37 for another 20 min (inhibition assay). Cells were then lysed in 250 μl sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCL, 2% w/v SDS, 10% glycerol, 50 mM dithiothreitol and 0.01% w/v bromophenol blue). The cell lysates obtained were sonicated for 5 sec at 4$^{o}$C and resolved in 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels and electroblotted to polyvinylidene difluoride membranes (PVDF; Bio-Rad, Hercules, CA). The membranes were blocked with 5% skim milk for 30 min and then probed with a rabbit anti-human phospho-ERK1/2 antibody (Cell Signaling, Danvers, MA; 1 : 2000), rabbit anti-human total ERK1/2 antibody (Cell Signaling, 1 : 2000), or rabbit anti-human β-actin antibody (Sigma, 1 : 250). After being washed, the membranes were incubated for 1 h at room temperature with an anti-rabbit IgG antibody conjugated with horseradish peroxidase (R&D Systems, Minneapolis, MN; 1 : 2000) in Tris-buffered saline-T (TBS; 20 mM Tris-HCL, 0.15 M NaCl, pH 7.6). After further washing, immunodetection was performed using ECL Prime Western blotting detection reagents (GE Healthcare, Little Chalfont, UK), and band densities were measured by ImageJ, a java-based image processing software (NIH, Bethesda, MD).

*2.5. Statistical Analysis*

Differences between two groups of interest were analyzed by the Student's *t*-test and were considered significant for p-values less than 0.05.

## 3. Results

### *3.1. VEGF Expression*

The LL37 treatment significantly increased VEGF mRNA expression in HP1 cells, with a peak being observed at 3 h (Fig. 1A). The up-regulated expression of VEGF mRNA was also noted in both HP2 and HP3 cells following the treatment with LL37 (Fig. 1A). These increases in all cells were dose-dependent up to the maximum dose examined, i.e., 10 μg/ml LL37 (Fig. 1B).

Consistent with the VEGF mRNA expression results, VEGF protein levels measured by ELISA increased in a time-dependent manner with the LL37 treatment in the representative HP2 cells (Fig. 2A). Although LL37 failed to significantly increase VEGF production at lower concentrations, it did so at 10 μg/ml (Fig. 2B).

### *3.2. The ERK Signaling Pathway in LL37-induced VEGF Production*

The inhibition assay showed that the ERK kinase inhibitor significantly attenuated LL37-induced VEGF mRNA expression in all tested cells, while the other inhibitors did not (Fig. 3A). LL37-induced VEGF protein production in the representative HP2 cells was also consistently abolished by the ERK kinase inhibitor (Fig. 3B).

As expected from the above results, LL37 induced the phosphorylation of ERK1/2 in the representative HP2 cells, with the maximal effect being observed 20 min after the LL37 treatment (Fig. 4). However, the ERK kinase inhibitor, which suppressed LL37-induced VEGF expression, clearly blocked LL37-induced ERK1/2 phosphorylation (Fig. 5A & 5B).

## 4. Discussion

The present study demonstrated that LL37 enhanced the secretion of VEGF from HP cells. Since VEGF is known to be a very potent angiogenic factor, an increase in its level will promote angiogenesis, which in turn may facilitate pulp wound healing. VEGF was also shown to induce the migration, proliferation and differentiation of HP cells, mediated by the kinase domain-containing receptor (KDR/VEGFR-2) and activator protein 1 (AP-1) depending signaling pathway (43). Thus, with these extra functions, LL37-stimulated VEGF in HP cells is thought to contribute more significantly to the pulp wound healing process.

LL37 has been reported to induce angiogenesis by directly activating endothelial cells without stimulating the release of VEGF (39). However, LL37 was shown to induce the release of VEGF from HP cells in the present study, which indicates that LL37 also has an indirect angiogenic effect, making it an even more potent inducer of angiogenesis. In other words, particularly in the context of the dental pulp, which is a highly vascularized tissue and mainly contains pulp fibroblasts expressing VEGF (37), LL37 may act on both endothelial cells and pulp cells, and its application to pulp injury or amputated pulp may effectively promote angiogenesis and accelerate the healing process.

The present study showed that LL37-induced VEGF expression was significantly suppressed by the ERK kinase inhibitor (Fig. 3). This result suggests that LL37 stimulates VEGF release from HP cells through the ERK signaling pathway. LL37 not only activates ERK, but also NF-κB to promote the expression of VEGF in human periodontal ligament cells (44). However, the NF-κB inhibitor failed to block LL37-induced VEGF mRNA expression in HP cells in the present study (Fig. 3A).

Therefore, the mechanisms responsible for LL37-induced VEGF expression may differ from cell to cell.

LL37 is known to activate/transactivate a number of cell-surface receptors, such as the formyl peptide receptor-like 1 (FPRL1), the $P2X_7$ receptor, the epidermal growth factor receptor (EGFR), the insulin-like growth factor 1 receptor (IGF-1R) and other still-uncharacterized G-protein coupled receptors (GPCR) (45-49). A previous study has shown that LL37 transactivates the EGFR to induce JNK phosphorylation, which results in the induction of HP cell migration (19). However, the results of the present study demonstrated that the JNK signaling cascade was independent to LL37-induced VEGF mRNA expression (Fig. 3A). In addition, the pretreatment with an EGFR neutralizing antibody failed to inhibit either LL37-induced VEGF mRNA expression or ERK phosphorylation in HP cells (data not shown), which suggests that EGFR transactivation is not involved in this case. Similarly, neither an IGF-1R neutralizing antibody nor an antagonist of FPRL1 suppressed the VEGF expression induced by LL37 (data not shown). Thus, at present, it is speculated that LL37-induced VEGF expression in HP cells may be mediated by a GPCR, which remains to be identified.

In summary, LL37 activates the ERK signaling pathway to boost the secretion of VEGF from HP cells.

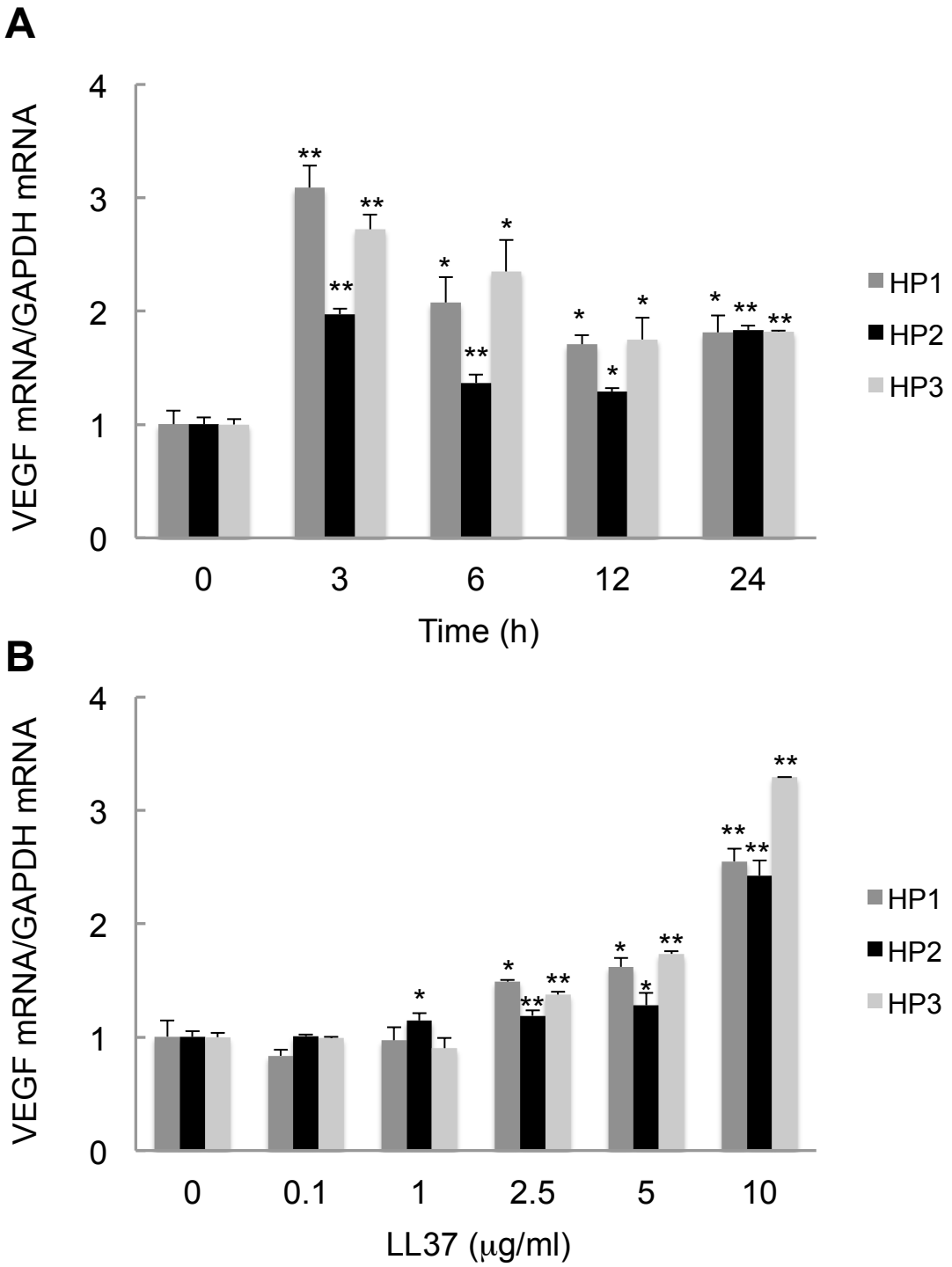


**Figure 1.** The effects of LL37 on VEGF mRNA expression in HP cells. (A and B) The relative ratio of VEGF mRNA levels to those of GAPDH determined by real-time PCR. HP1, HP2, and HP3 cells were stimulated with 10 μg/ml LL37 for the indicated periods of time before the end of the incubation on day 8 (A), or with the indicated doses of LL37 for 3 h (B). The data shown are the representatives of three independent experiments with similar results, and values are the means ± standard deviation of triplicate determinations. *$p < 0.05$, **$p < 0.01$ vs. controls (no exposure to LL37)

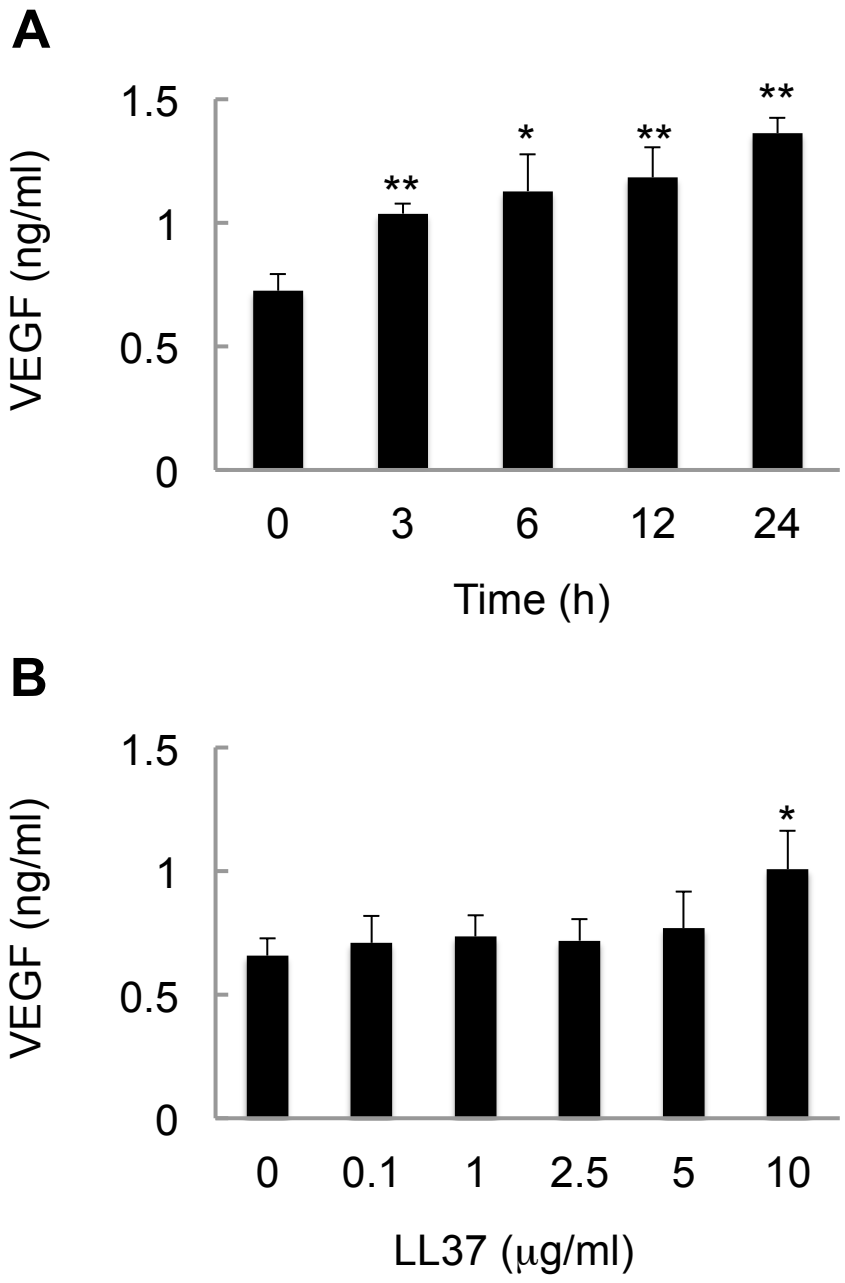


**Figure 2.** The effects of LL37 on VEGF protein production in HP cells. (A and B) VEGF protein levels measured by ELISA. The representative HP2 cells were stimulated with 10 μg/ml LL37 for the indicated periods of time before the end of the incubation on day 8 (A), or with the indicated doses of LL37 for 24 h (B). The data shown are the representatives of two independent experiments with similar results, and values are the means ± standard deviation of triplicate determinations. *$p < 0.05$, **$p < 0.01$.

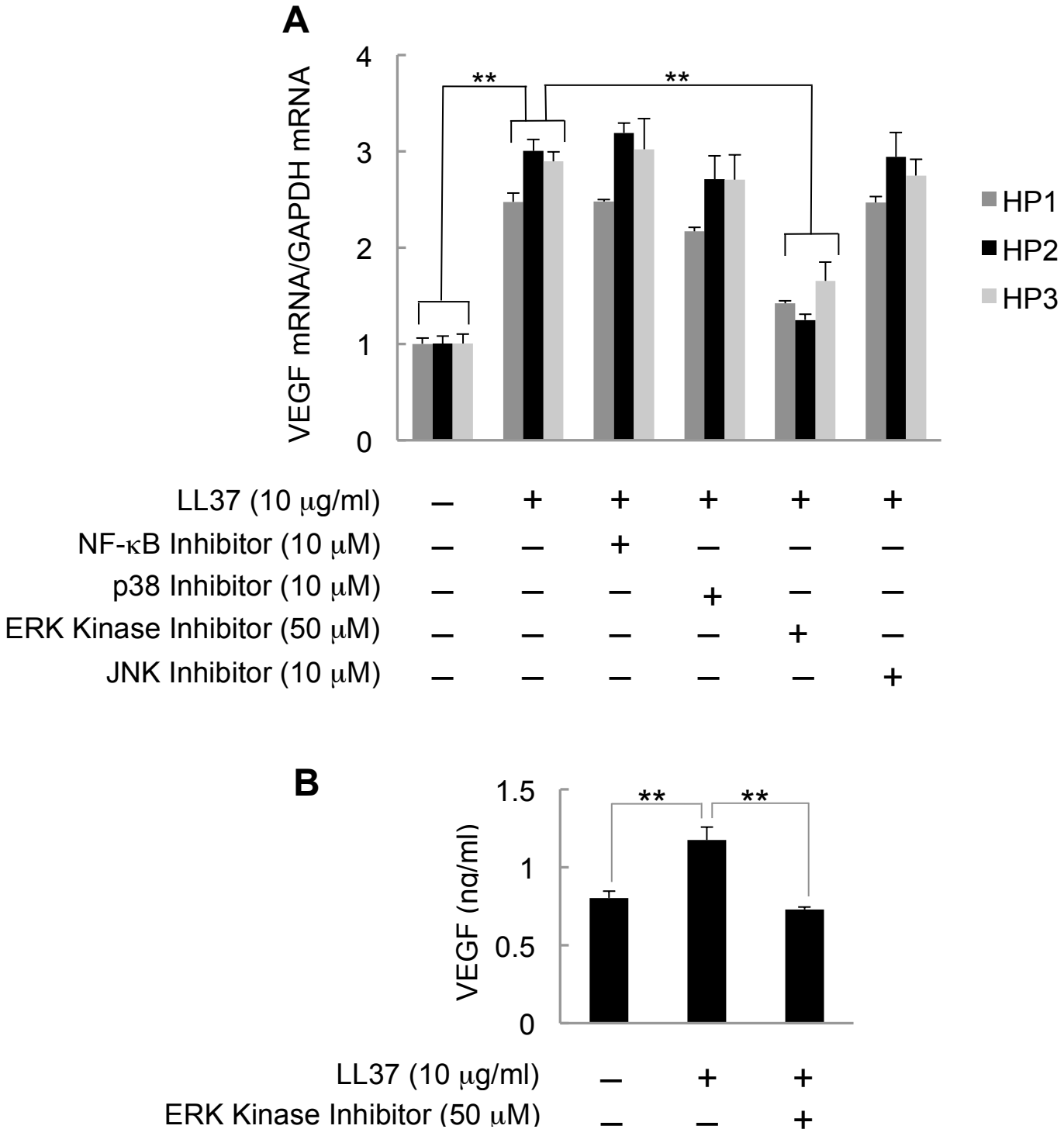


**Figure 3.** The involvement of the ERK pathway in LL37-induced VEGF expression in HP cells. (A) The relative ratio of VEGF mRNA levels to those of GAPDH. Cells were either pretreated or not for 30 min with the inhibitors and stimulated with LL37 for another 3 h. (B) VEGF protein levels. The representative HP2 cells were either pretreated or not for 30 min with the ERK kinase inhibitor before the exposure to LL37 for 24 h. The data shown are the representatives of two independent experiments with similar results, and values are the means ± standard deviation of triplicate determinations. **$p < 0.01$: differs significantly among different treatments in each cell line.

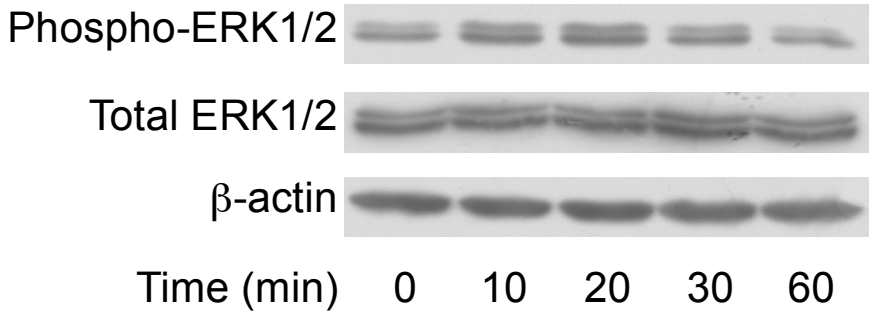


**Figure 4.** The effect of LL37 on phosphorylated ERK1/2 levels. The representative HP2 cells were treated with 10 μg/ml LL37 for the indicated periods of time before the end of the incubation. The representative blots of phosphorylated ERK1/2, total ERK1/2, and β-actin from three independent experiments with similar results are presented.

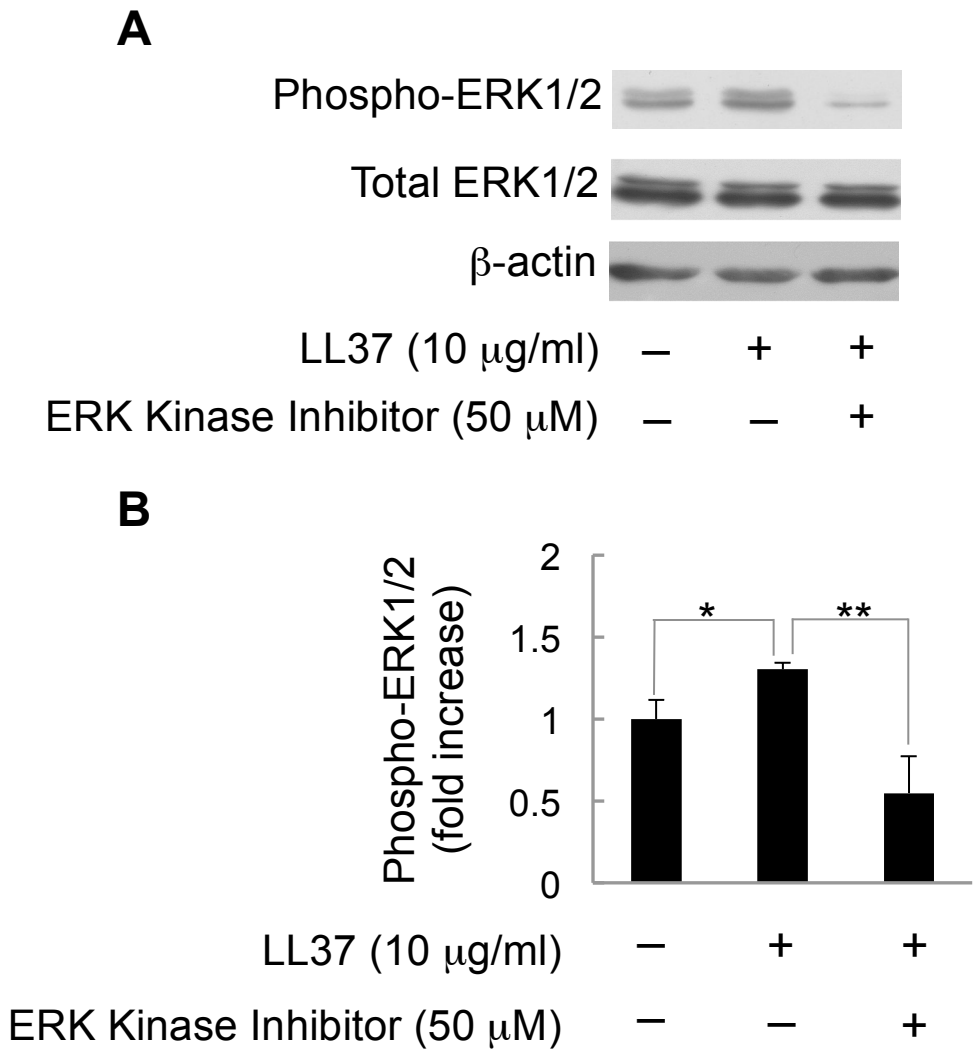


**Figure 5.** The effect of the ERK kinase inhibitor on LL37-induced phosphorylated ERK1/2 levels. HP2 cells were either pretreated or not with the ERK kinase inhibitor (50 μM) for 30 min before the exposure to LL37 (10 μg/ml) for another 20 min. The representative blots of phosphorylated ERK1/2, total ERK1/2, and β-actin from three independent experiments with similar results are presented (A), while the corresponding graph shows the mean densities ± standard deviation of phospho-ERK1/2 bands from the three experiments measured by ImageJ software (B). The band density of untreated samples was set a value of 1, and those of treated ones (i.e., LL37 ± ERK kinase inhibitor) were normalized to this. *$p < 0.05$, and **$p < 0.01$.

# Chapter Three

# The Inhibition/Reduction of Pulp Tissue Breakdown by LL37

## 1. Introduction

Like any other inflammation, pulpitis is associated with tissue destruction, which is characterized by the degradation of extracellular matrix (ECM). At least three pathways have been recognized in the ECM degradation, that is, plasminogen (Plg)-dependent pathway, polymorphonuclear (PMN) leukocyte serine proteinase-dependent pathway and matrix metalloproteinase (MMP)-dependent pathway (50).

MMPs or matrixins are a family of zinc-dependent endopeptidases, which have a common zinc-binding motif in their active site followed by a conserved methionine turn. This MMP family contains at least 23 members in human, as listed in Table 1. Based on their substrate specificity and homology, these MMPs are divided into six groups: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs (MT-MMPs) and other MMPs, but together they are capable of degrading all kinds of ECM components (51). They also act on a variety of other substrates, such as other proteinases, latent growth factors, cell surface receptors and chemotactic molecules (52, 53). Due to their wide range of proteolytic activities, MMPs are involved in many physiological and pathological processes, including embryogenesis, normal tissue remodeling, wound healing, angiogenesis, cancer and inflammation (54-56).

The wide-scale expressions of MMPs and the tissue inhibitors of metalloproteinases (TIMPs) were observed in both human odontoblasts and pulp tissue, and they are considered to play a role in the formation and maintenance of the dentin-pulp complex by participating in dentin matrix organization prior to

mineralization (57). As an example, MMP-13 is highly expressed in human dental pulp (58) and particularly in long-term cultures of HP cells under the condition that support mineralization *in vitro* (59), suggesting its role in HP cell differentiation. In contrast, collagenolytic and elastinolytic activities were found in necrotic pulp while neither enzymatic activity was seen in healthy pulp (60), which indicates that MMP activities may also be involved in the pathological processes of pulp tissue. Indeed, the elevated levels of some MMPs have been reported in either inflamed pulps or cultures of pulp cells stimulated with inflammatory cytokines or bacteria. The concentrations of MMP-1 were significantly increased in acute and chronic inflamed pulps, while those of MMP-2 and MMP-3 were elevated in acute pulpitis (61). The higher levels of MMP-9 and gelatinolytic activity were also detected in inflamed pulps compared with healthy counterparts (62). Interleukin (IL)-1 or tumor necrosis factor (TNF)-α were shown to stimulate the production of MMP-2 and MMP-9 by HP cells during long-term culture (63). Similarly, other studies have reported the ability of IL-1α to induce the secretion of MMP-1 and MMP-3 from HP cells and the collagen degradation mediated by these cells (64, 65). Black-pigmented *Bacteroides* species (*Porphyromonas endodontalis* and *Porphyromonas gingivalis*) were capable of elevating the levels of MMP-2 but not those of MMP-9 in both the conditioned medium and cell extracts from long-term cultures of HP cells (66). Based on these findings that the expressions of MMPs, particularly those of MMP-1, MMP-2 and MMP-3, are increased in the inflammatory conditions of either pulp tissue or cells, it is likely that MMPs play a role in the tissue destruction of inflamed dental pulp.

Inflammation is usually initiated as a response to bacteria or bacterial components, such as peptidoglycan (PGN). PGN is a polymer consisting of sugars and amino acids that forms a mesh-like layer outside the plasma membrane of

bacteria, forming the cell wall. It is possessed by virtually all bacteria (except Mycoplasma), but its amount, location and specific composition vary, depending on bacteria types. In Gram-positive bacteria, PGN is found as a thick exposed layer in association with LTA, whereas in Gram-negative bacteria it is present as a thin layer overlaid by LPS. The sugar component of PGN consists of alternating residues of β-(1,4) linked of *N*-acetylglucosamine and *N*-acetylmuramic acid, which are cross-linked by short peptide chains called stem peptides. Based on the nature of the third residue of these stem peptides, PGN can be classified into two major types, that is, DAP-PGN in Gram-negative bacteria and Lys-PGN in Gram-positive bacteria (67). PGN is an immunostimulatory component of the bacterial cell wall and has been demonstrated to stimulate the production of inflammatory cytokines, such as IL-6, IL-8, IL-1α/β and TNF-α in eosinophils, macrophages and epithelial cells (68–70). Interestingly, it also increases IL-6 levels in HP cells (71). Thus, PGN may induce the inflammatory conditions of dental pulp, which are probably associated with the elevated levels of MMPs.

LL37 is known to inhibit the immunostimulatory activities of bacteria-derived molecules. For instance, LL37 suppressed LPS-induced TNF-α mRNA and protein expression in the murine macrophage cell line RAW 264.7 (72). Microarray studies also demonstrated that 106 out of 125 genes up-regulated by LPS in human monocytes were suppressed in the presence of LL37 (73). Consistent with these observations, monocyte-derived dendritic cells stimulated by LPS, LTA or flagellin in the presence of LL37 released lower amounts of IL-6, IL-12 and TNF-α (74). In addition, LL37 was reported to abolish the mRNA expressions of IL-6 and IL-8 induced by PGN in HP cells (75).

Therefore, to study the capability of LL37 to inhibit or reduce pulp tissue breakdown, its effects on the expressions of MMPs, mainly those of MMP-1, MMP-2 and MMP-3, in PGN-stimulated HP cells were examined.

*Table 1. Human Matrix Metalloproteinases*

| Types | MMPs | Substrates |
|---|---|---|
| Collagenases | MMP-1 | Type I, II, III, VII, VIII, X, XI collagens, gelatin, fibronectic, laminin, tenascin, $\alpha_2$-macroglobulin, IL-1β, proTNF-α, proMMP-1, -2 and -9 |
| | MMP-8 | Type I, II, III collagen, aggrecan, fibrinogen, $\alpha_2$-macroglobulin, bradykinin |
| | MMP-13 | Type I, II, III, IV, VI, IX, X, XIV collagens, collagen telopeptides, gelatin, fibronectin, tenascin-C, aggrecan, fibrinogen, $\alpha_2$-macroglobulin, proMMP-9 |
| Gelatinases | MMP-2 | Type I, II, III, IV, V, VII, X, XI collagens, gelatin, laminin, elastin, fibronectin, $\alpha_2$-macroglobulin, IL-1b, proTNF-α, latent TGF-β, proMMP-1, -2, -9 and -13 |
| | MMP-9 | Type IV, V, XI, XIV collagens, gelatin, elastin, laminin, aggrecan, $\alpha_2$-macroglobulin, IL-1β, proTNF-α |
| Stromelysins | MMP-3 | Type III, IV, V, VII, IX, X, XI collagens, collagen telopeptides, gelatin, elastin, fibronectin, laminin, aggrecan, decorin, perlecan, versican, $\alpha_2$-macroglobulin, IL-1β, proTNF-α, fibrinogen, proMMP-1, -3, -7, -8, -9 and -13 |
| | MMP-10 | Type III, IV, V collagens, gelatin, elastin, fibronectin, aggrecan, proMMP-1, -7, -8 and -9 |
| | MMP-11 | Type IV collagen, gelatin, fibronectin, $\alpha_2$-proteinase inhibitor |
| Matrilysins | MMP-7 | Type I, IV collagens, gelatin, elastin, fibronectin, laminin, aggrecan, $\alpha_2$-macroglobulin, proMMP-1, -2, -7 and -9 |
| | MMP-26 | Gelatin, fibronectin, $\alpha_2$-macroglobulin, fibrinogen, proMMP-9 |
| MT-MMPs | MMP-14 | Type I, II, III collagens, gelatin, fibronectin, laminin, aggrecan, $\alpha_2$-macroglobulin, proTNF-α, fibrinogen, proMMP-2, -13 and -20 |
| | MMP-15 | Fibronectin, tenascin, laminin, aggrecan, proTNF-α, proMMP-2 |
| | MMP-16 | Type III collagen, gelatin, fibronectin, laminin, $\alpha_2$-macroglobulin, proMMP-2 |
| | MMP-17 | Gelatin, fibrinogen, fibrin, proTNF-α |
| | MMP–24 | Fibronectin, gelatin, chondroitin sulphate proteoglycan, proMMP-2 |
| | MMP-25 | Type IV collagen, gelatin, fibronectin, fibrinogen, fibrin, proMMP-2 |
| Other MMPs | MMP-12 | Type I, IV, V collagens, elastin, gelatin, fibronectin, laminin, aggrecan, $\alpha_2$-macroglobulin, proTNF-α, fibrinogen |

*Table 1. Continued.*

| Type | MMP | Substrates |
|---|---|---|
| | MMP-19 | Type IV collagen, gelatin, laminin, fibronectin, fibrinogen, fibrin |
| | MMP-20 | Amelogenin, type IV collagen, aggrecan, fibronectin, laminin, tenascin-C |
| | MMP-21 | α1-antitrypsin |
| | MMP-23 | Gelatin |
| | MMP-27 | – |
| | MMP-28 | Casein |

Adapted from (51).

## 2. Materials and Methods

### *2.1. Cell Culture*

The representative HP2 cells were used and cultured until they were ready for experiments, as described in the section *2.1.* of chapter two.

### *2.2. RNA Preparation*

HP2 cells were stimulated with 10 μg/ml PGN (derived from *Staphylococcus aureus*; Sigma) for various lengths of time (0-24 h) before the end of the incubation on day 8. To study the involvement of LL37 and the intracellular signaling pathway in the PGN effect on MMP expressions, the cells were either pretreated or not for 30 min with 10 μg/ml LL37 or 10 μM of each inhibitor (same as those used in the VEGF study) and stimulated for another 24 h with 10 μg/ml PGN. Total RNA was isolated, purified, quantified and reverse transcribed as described in the section *2.2.* of chapter two.

*2.3. Real-time PCR*

mRNA expressions of MMP-1, MMP-2 and MMP-3 were quantified by real-time PCR. The PCR was carried out in two steps with a Lightcycler system using SYBR green (Roche Diagnostics, Mannheim, Germany). The sense primers and antisense primers used to detect the mRNA of GAPDH, MMP-1, MMP-2 and MMP-3 are listed in Table 2.

*Table 2. Sense and Antisense Primers for Real-time PCR*

| Target Gene | | Primer Sequence |
|---|---|---|
| GAPDH | Forward | 5′-AACGTGTCAGTGGTGGACCTG-3′ |
| | Reverse | 5′-AGTGGGTGTCGCTGTTGAAGT-3′ |
| MMP-1 | Forward | 5′-CTGGCCACAACTGCCAAATG-3′ |
| | Reverse | 5′-CTGTCCCTGAACAGCCCAGTACTTA-3′ |
| MMP-2 | Forward | 5′-TCTCCTGACATTGACCTTGGC-3′ |
| | Reverse | 5′-CAAGGTGCTGGCTGAGTAGATC-3′ |
| MMP-3 | Forward | 5′-ATTCCATGGAGCCAGGCTTTC-3′ |
| | Reverse | 5′-CATTTGGGTCAAACTCCAACTGTG-3′ |

*2.4. p38 Phosphorylation and Total p38 Expression*

HP2 cells were stimulated with 10 μg/ml PGN for 0-60 min before the end of the incubation, or were either pretreated or not for 30 min with 10 μg/ml LL37 before being exposed to 10 μg/ml PGN for another 30 min. The cells were then handled as described in the section *2.4*. of chapter two. Samples were resolved in 10% SDS-PAGE gels and electroblotted to PVDF membranes, which were subsequently blocked with 5% skim milk for 1 h and then probed with a rabbit anti-human phospho-p38 antibody (Cell Signaling, 1 : 1000) or rabbit anti-human total p38 antibody (Cell Signaling, 1 : 1000). After being washed, the membranes were

incubated for 1 h at room temperature with an anti-rabbit IgG antibody conjugated with horseradish peroxidase, and immunodetection was performed using ECL Prime Western blotting detection reagents.

*2.5. Statistical Analysis*

The Student's *t*-test was used.

**3. Results**

As expected, PGN significantly increased the mRNA expressions of MMP-1, MMP-2 and MMP-3 in a time-dependent manner (Fig. 6). However, the pretreatment with LL37 abolished these increases (Fig. 7). Similarly, the p38 inhibitors also suppressed all the MMP mRNA expressions induced by PGN (Fig. 8). The ERK kinase inhibitor decreased the elevated mRNA levels of MMP-1 but not those of MMP-2 and MMP-3, while the JNK inhibitor decreased the elevated mRNA levels of MMP-1 and MMP-3 but not that of MMP-2 (Fig. 8). Although there were significant differences in MMP-1 or MMP-1 and MMP-3 mRNA levels between no treatment and pretreatment with the ERK kinase inhibitor or the JNK inhibitor, the decreases in MMP expressions caused by these two inhibitors were less pronounced than those caused by the p38 inhibitor (Fig. 8). In other words, the decreases in MMP expressions caused by the p38 inhibitor were the most pronounced. In contrast, the NF-κB inhibitor had no suppressive effect on all the MMP mRNA expressions induced by PGN at all (Fig. 8).

PGN induced the phosphorylation of p38, with the maximal effect being observed 30 min after the PGN treatment (Fig. 9). However, this increased level of phosphorylated p38 was apparently suppressed by LL37 (Fig. 10).

## 4. Discussion

The present study demonstrated that the p38 inhibitor most robustly blocked PGN-induced mRNA expressions of MMP-1, MMP-2 and MMP-3 in HP cells (Fig. 8), suggesting that PGN most likely activates p38 to increase these MMP mRNA levels in HP cells. This finding is consistent with a previous study, which has reported that PGN induces IL-6 levels in HP cells through the p38 pathway (71). However, PGN is known to induce inflammatory cytokine productions through the activation of a number of signaling pathways in various cell types. For example, PGN induces the release of IL-1β, IL-6 and IL-8 via NF-κB and ERK in human eosinophils (68); it boosts IL-6 production in the murine macrophage cell line RAW 264.7 through p38 and NF-κB (69); and it stimulates multiple signaling pathways, including NF-κB, p38 and JNK to enhance the secretions of IL-6, IL-8 and TNF-α in human corneal epithelial cells (70). Thus, it is noticeable that the intracellular signaling molecules involved in the responses elicited by PGN seem to vary, depending on the cell type being examined.

LL37 was shown to suppress both PGN-induced MMP expressions and PGN-induced p38 phosphorylation in the present study (Fig. 7 & Fig. 10). These findings strongly indicate that LL37 decreases PGN-induced MMP expressions by blocking the phosphorylation of p38. Still, the exact mechanism is yet to be identified. Nevertheless, a previous study has reported that LL37 inhibits IL-6 and IL-8 mRNA expressions induced by PGN in HP cells possibly through the $P2X_7$ receptor (75). Therefore, it is reasonable to assume that LL37 probably interacts with the $P2X_7$ receptor, resulting in the suppression of p38 phosphorylation, which in turn leads to the abrogation of the increases in MMP expressions induced by PGN.

The extracellular matrix of the dental pulp is composed mainly of collagens type I and III (76), and the degradation of these collagens is believed to play a role in the tissue destruction during pulp inflammation. MMP-1 is a key enzyme involved in degrading the triple helix of collagen types I and III (51), and MMP-2 and MMP-3 may also degrade these collagens as outlined in Table 1. In addition, the increased levels of these MMPs have been reported to be observed in inflamed pulps (61). Therefore, they are suggested to be involved in pulp tissue destruction. Here, LL37 was shown to suppress the increases in these MMP mRNA levels induced by PGN in HP cells, so their suppression by LL37 means that LL37 can inhibit or at least counter the breakdown of pulp tissue.

As noted earlier, MMPs participate in a wide range of physiological and pathological processes, including angiogenesis. During angiogenic process, the endothelial cells activated by angiogenic factors release proteases, such as MMPs, to degrade the basement membrane so that they can migrate and proliferate into the surrounding matrix (77). Here, LL37 was shown to suppress the mRNA expressions of MMP-1, MMP-2 and MMP-3 induced by PGN in HP cells, thus raising a concern whether or not the suppressive effect of LL37 on the MMP expressions may impair the angiogenic role of MMPs. Fortunately, this is unlikely because MMPs, which are necessary for the angiogenic process, can be released by the activated endothelial cells themselves (77). Thus, the suppression of PGN-induced MMP expressions in HP cells by LL37 may not interfere with the angiogenic activity of those MMPs released by endothelial cells.

The dental pulp is known to have a good reparative/regenerative potential once certain prerequisites are met, including the elimination of bacteria, regulation of inflammation, promotion of pulp cell function, and angiogenesis. Previous studies

have demonstrated that LL37 is effective against cariogenic bacteria (18) and induces the migration of HP cells (19). The present study additionally showed that LL37 increased VEGF expression and abolished PGN-induced MMP expressions in HP cells. These two additional effects of LL37 on HP cells should in turn promote angiogenesis and inhibit pulp tissue breakdown during pulpal inflammation, respectively. Although it remains to determine whether LL37 can stimulate reparative dentin formation, a recent study has reported that LL37 promotes bone regeneration in a rat calvarial bone defect (78), suggesting that this peptide may also possess osteogenic/odontogenic induction activity. Based on these findings, LL37 is strongly proposed as an agent to facilitate pulp wound healing and may be used as a pulp capping material. In addition, all three HP cells tested in this study showed an up-regulated level of VEGF following the LL37 treatment. This consistent effect of LL37 on HP cells from different individuals may indicate its effectiveness when applied to the general population.

In summary, PGN increases the mRNA expression of MMP-1, MMP-2 and MMP-3 through the activation of p38. However, LL37 suppresses this effect, resulting in the decrease in these MMP mRNA levels induced by PGN.

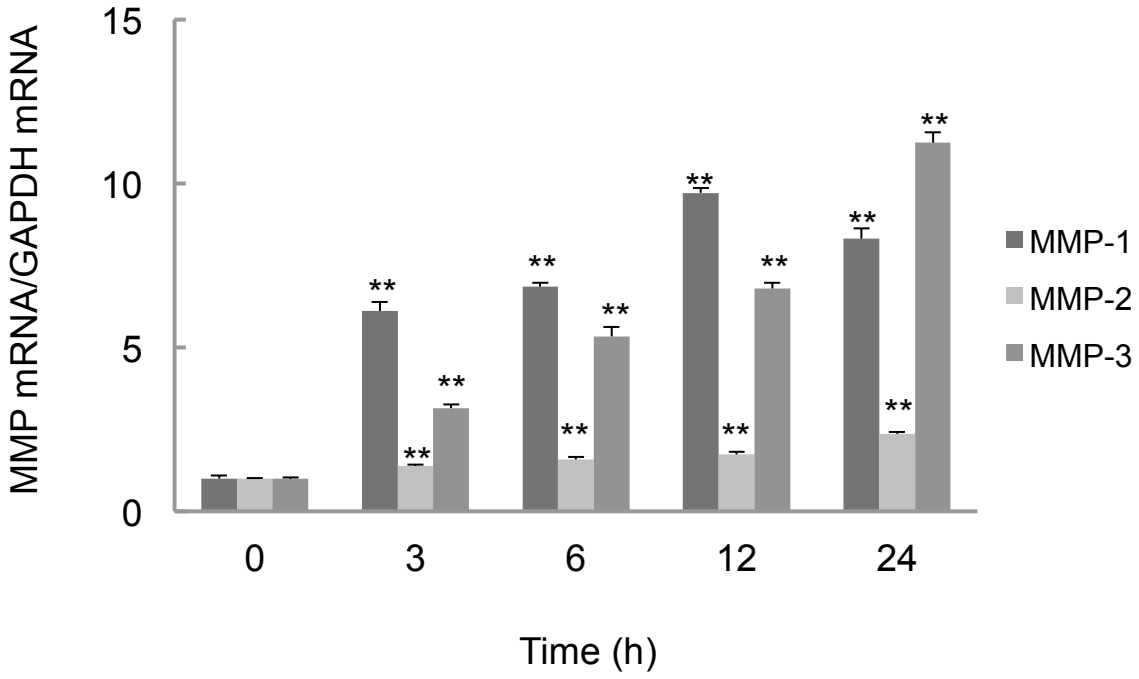


**Figure 6.** The effect of PGN on MMP mRNA expressions in HP cells. HP2 cells were stimulated with 10 μg/ml PGN for the indicated periods of time before the end of the incubation on day 8. The graph shows the relative ratio of MMP-1, MMP-2 and MMP-3 mRNA levels to those of GAPDH determined by real-time PCR. Data are the representatives of two independent experiments with similar results, and values are the means ± standard deviation of triplicate determinations. **$p < 0.01$ vs. controls (no exposure to PGN).

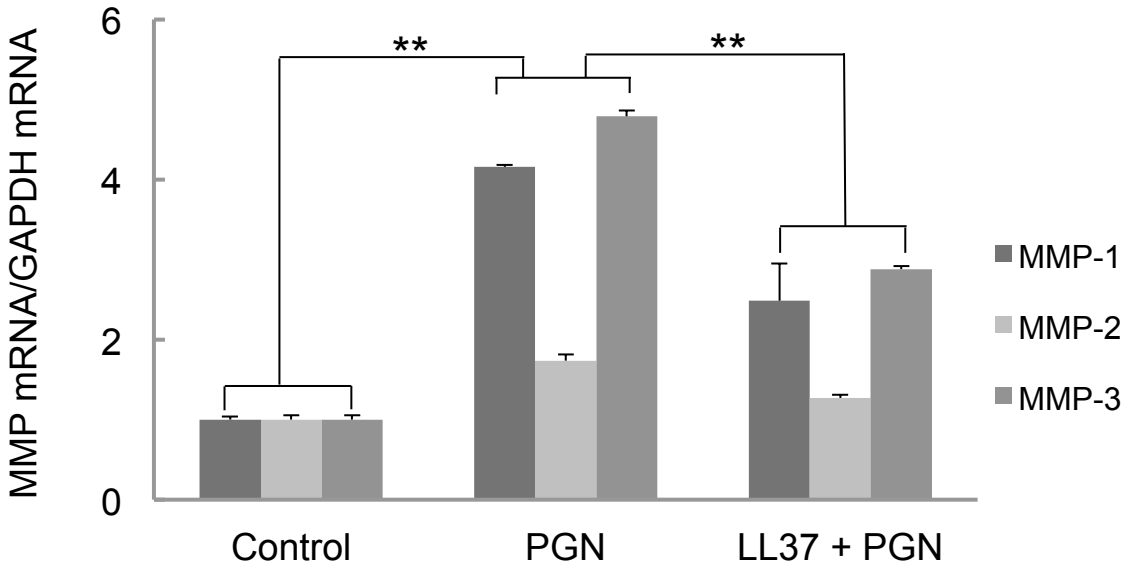


**Figure 7.** The effect of LL37 on PGN-induced MMP mRNA expressions in HP cells. HP2 cells were either pretreated or not for 30 min with 10 μg/ml LL37 before the exposure to 10 μg/ml PGN for another 24 h. The graph shows the relative ratio of MMP-1, MMP-2 and MMP-3 mRNA levels to those of GAPDH determined by real-time PCR. Data are the representatives of three independent experiments with similar results, and values are the means ± standard deviation of triplicate determinations. **$p < 0.01$: differs significantly among each MMP.

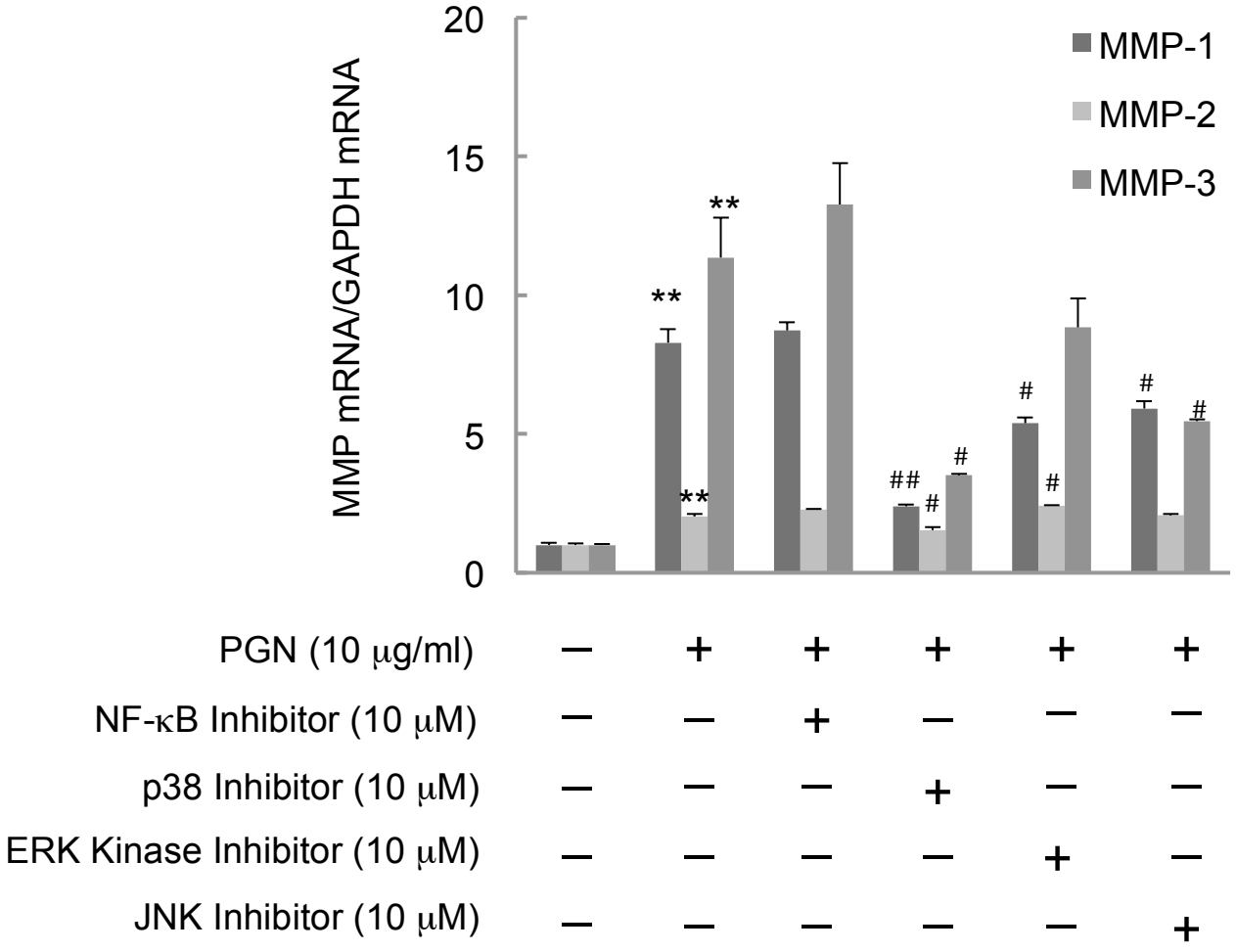


**Figure 8.** The involvement of the p38 pathway in PGN-induced MMP expressions in HP cells. The graph shows the relative ratio of MMP-1, MMP-2 and MMP-3 mRNA levels to those of GAPDH. HP2 cells were either pretreated or not for 30 min with the inhibitors and stimulated with PGN for another 24 h. Data shown are the representatives of three independent experiments with similar results, and values are the means ± standard deviation of triplicate determinations. $^{**}p < 0.01$ vs. controls (no exposure to PGN) & $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. PGN-treated for each MMP.

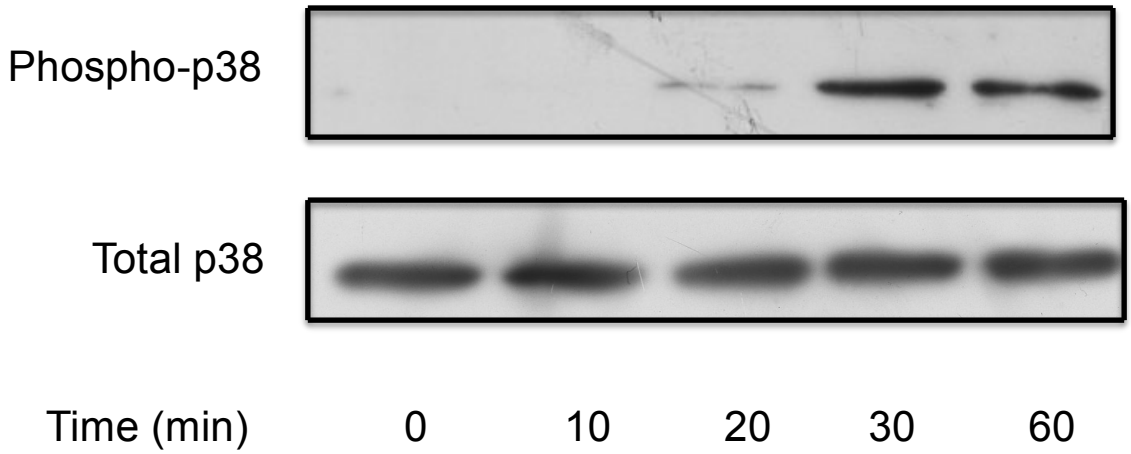


**Figure 9.** The effect of PGN on phosphorylated p38 levels. HP2 cells were treated with 10 μg/ml PGN for the indicated periods of time before the end of the incubation. The representative blots of phosphorylated p38 and total p38 from three independent experiments with similar results are presented.

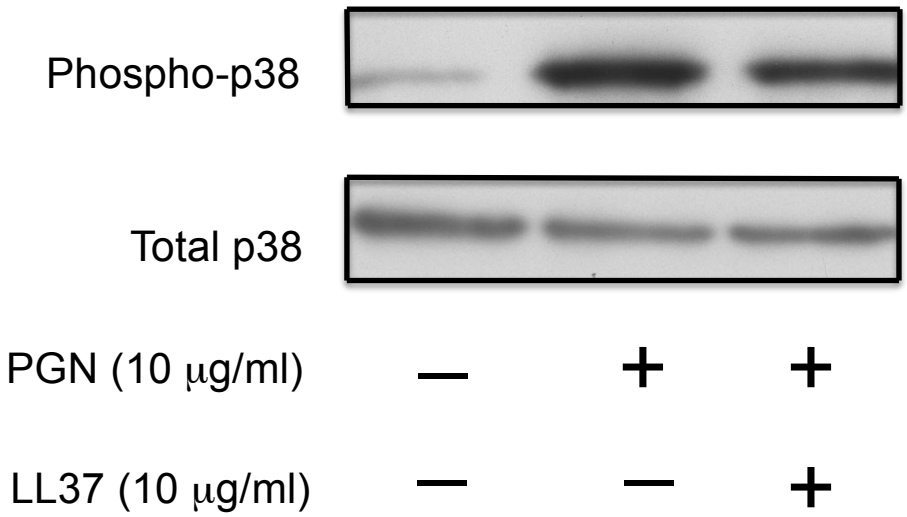


**Figure 10.** The effect of LL37 on PGN-induced phosphorylated p38 levels. HP2 cells were either pretreated or not with 10 μg/ml LL37 for 30 min before the exposure to 10 μg/ml PGN for another 30 min. The representative blots of phosphorylated p38 and total p38 from three independent experiments with similar results are presented

# Chapter Four

## Summary and Conclusion

The objective of this work was to study the possible application of LL37 as a pulp capping by focusing on two main aspects: the angiogenic effect of LL37 on pulp cells and the inhibition/reduction of pulp tissue breakdown by LL37. As a result, the following findings were determined:

1. LL37 activates ERK to boost VEGF secretion from HP cells, and this may induce angiogenesis through the VEGF released.
2. LL37 suppresses the increases in MMP-1, MMP-2 and MMP-3 mRNA levels induced by PGN in HP cells through the p38 signaling pathway, suggesting that LL37 may be able inhibit or at least reduce pulp tissue destruction caused by these MMPs.

Thus, based on the findings of the present study and those of previous ones that LL37 is effective against cariogenic bacteria (18) and induces pulp cell migration (19), it is reasonable to conclude that LL37 is a promising candidate for a pulp capping material. However, many challenges remain to be overcome before the actual transition of this peptide from the laboratory into the clinic, including its effectiveness to stimulate pulp wound healing and reparative dentin formation in the *in vivo* settings, the concern over its known cytotoxicity, its application methods, and finally the costs of its production. Despite these challenges, further investigations on its clinical application for this purpose are warranted.

# References


1. Pissiotis E, Spångberg LS. Dentin permeability to bacterial proteins in vitro. J Endod 1994;20:118-122.

2. Dommisch H, Winter J, Açil Y, Dunsche A, Tiemann M, Jepsen S. Human beta-defensin (hBD-1, -2) expression in dental pulp. Oral Microbiol Immunol 2005;20:163-166.

3. Randow K, Glantz PO. On cantilever loading of vital and non-vital teeth. An experimental clinical study. Acta Odontol Scand 1986;44:271-277.

4. Caplan DJ, Cai J, Yin G, White BA. Root canal filled versus non-root canal filled teeth: a retrospective comparison of survival times. J Public Health Dent 2005;65:90-96.

5. American Association of Endodontists. *Glossary of Endodontic Terms*. Chicago, IL: American Association of Endodontists;2003:40.

6. Al-Hiyasat AS, Barrieshi-Nusair KM, Al-Omari MA. The radiographic outcomes of direct pulp-capping procedures performed by dental students: a retrospective study. J Am Dent Assoc 2006;137:1699-1705.

7. Hilton TJ. Keys to clinical success with pulp capping: a review of the literature. Oper Dent 2009;34:615-625.

8. Zanetti M, Gennaro R, Romeo D. Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. FEBS Lett 1995;374:1-5.

9. Larrick JW, Hirata M, Balint RF, Lee J, Zhong J, Wright SC. Human CAP18: a novel antimicrobial lipopolysaccharide-binding protein. Infect Immun 1995;63:1291-1297.

10. Zanetti M. Cathelicidins, multifunctional peptides of the innate immunity. J Leukoc Biol 2004;75:39-48.

11. Cowland JB, Johnsen AH, Borregaard N. hCAP-18, a cathelin/pro-bactenecin-like protein of human neutrophil specific granules. FEBS Lett 1995;368:173-176.

12. Agerberth B, Charo J, Werr J, Olsson B, Idali F, Lindbom L, Kiessling R, Jörnvall H, Wigzell H, Gudmundsson GH. The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations. Blood 2000;96:3086-3093.

13. Frohm M, Agerberth B, Ahangari G, Ståhle-Bäckdahl M, Lidén S, Wigzell H, Gudmundsson GH. The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders. J Biol Chem 1997;272:15258-15263.

14. Bals R, Wang X, Zasloff M, Wilson JM. The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface. Proc Natl Acad Sci U S A 1998;95:9541-9546.

15. Sørensen O, Cowland JB, Askaa J, Borregaard N. An ELISA for hCAP-18, the cathelicidin present in human neutrophils and plasma. J Immunol Methods 1997;206:53-59.

16. Radek K, Gallo R. Antimicrobial peptides: natural effectors of the innate immune system. Semin Immunopathol 2007;29:27-43.

17. Bucki R, Leszczyńska K, Namiot A, Sokolowski W. Cathelicidin LL-37: a multitask antimicrobial peptide. Arch Immunol Ther Exp 2010;58:15-25.

18. Ouhara K, Komatsuzawa H, Yamada S, Shiba H, Fujiwara T, Ohara M, Sayama K, Hashimoto K, Kurihara H, Sugai M. Susceptibilities of periodontopathogenic and cariogenic bacteria to antibacterial peptides, β-defensins and LL37, produced by human epithelial cells. J Antimicrob Chemother 2005;55:888-896.

19. Kajiya M, Shiba H, Komatsuzawa H, Ouhara K, Fujita T, Takeda K, Uchida Y, Mizuno N, Kawaguchi H, Kurihara H. The antimicrobial peptide LL37 induces the migration of human pulp cells: a possible adjunct for regenerative endodontics. J Endod 2010;36:1009-1113.

20. Tjäderhane L. The mechanism of pulpal wound healing. Aust Endod J 2002;28:68-74.

21. Nakashima M, Akamine A. The application of tissue engineering to regeneration of pulp and dentin in endodontics. J Endod 2005;31:711-718.

22. O'Connor DS, Schechner JS, Adida C, Mesri M, Rothermel AL, Li F, Nath AK, Pober JS, Altieri DC. Control of apoptosis during angiogenesis by survivin expression in endothelial cells. Am J Pathol 2000;156:393-398.

23. Vlodavsky I, Ishai-Michaeli R, Bashkin P, Levi E, Korner G, Bar-Shavit R, Fuks Z, Klagsbrun M. Extracellular matrix-resident basic fibroblast growth factor: implication for the control of angiogenesis. J Cell Biochem 1991;45:167-176.

24. Pintucci G, Froum S, Pinnell J, Mignatti P, Rafii S, Green D. Trophic effects of platelets on cultured endothelial cells are mediated by platelet-associated fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF). Thromb Haemost 2002;88:834-842.

25. Roskoski R Jr. Vascular endothelial growth factor (VEGF) signaling in tumor progression. Crit Rev Oncol Hematol 2007;62:179-213.

26. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 1983;219:983-985.

27. Connolly DT, Heuvelman DM, Nelson R, Olander JV, Eppley BL, Delfino JJ, Siegel NR, Leimgruber RM, Feder J. Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. J Clin Invest 1989;84:1470-1478.

28. Favard C, Moukadiri H, Dorey C, Praloran V, Plouët J. Purification and biological properties of vasculotropin, a new angiogenic cytokine. Biol Cell 1991;73:1-6.

29. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 2004;25:581-611.

30. Brown LF, Berse B, Tognazzi K, Manseau EJ, Van de Water L, Senger DR, Dvorak HF, Rosen S. Vascular permeability factor mRNA and protein expression in human kidney. Kidney Int 1992; 42:1457-1461.

31. Sharkey AM, Charnock-Jones DS, Boocock CA, Brown KD, Smith SK. Expression of mRNA for vascular endothelial growth factor in human placenta. J Reprod Fertil 1993;99:609-615.

32. Dvorak HF. Angiogenesis: update 2005. J Thromb Haemost 2005;3:1835-1842.

33. Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 1996;16:4604-4613.

34. Pertovaara L, Kaipainen A, Mustonen T, Orpana A, Ferrara N, Saksela O, Alitalo K. Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. J Biol Chem 1994;269:6271-6274.

35. Bermont L, Lamielle F, Lorchel F, Fauconnet S, Esumi H, Weisz A, Adessi GL. Insulin up-regulates vascular endothelial growth factor and stabilizes its messengers in endometrial adenocarcinoma cells. J Clin Endocrinol Metab 2001;86:363-368.

36. Artese L, Rubini C, Ferrero G, Fioroni M, Santinelli A, Piattelli A. Vascular endothelial growth factor (VEGF) expression in healthy and inflamed human dental pulps. J Endod 2002;28:20-23.

37. Tran-Hung L, Mathieu S, About I. Role of human pulp fibroblasts in angiogenesis. J Dent Res 2006;85:819-823.

38. Tran-Hung L, Laurent P, Camps J, About I. Quantification of angiogenic growth factors released by human dental cells after injury. Arch Oral Biol 2008;53:9-13.

39. Koczulla R, von Degenfeld G, Kupatt C, Krötz F, Zahler S, Gloe T, Issbrücker K, Unterberger P, Zaiou M, Lebherz C, Karl A, Raake P, Pfosser A, Boekstegers P, Welsch U, Hiemstra PS, Volgelmeier C, Gallo RL, Clauss M, Bals R. An angiogenic role for the human peptide antibiotic LL-37/hCAP-18. J Clin Invest 2003;111:1665-1672.

40. Khine AA, Del Sorbo L, Vaschetto R, Voglis S, Tullis E, Slutsky AS, Downey GP, Zhang H. Human neutrophil peptides induce interleukin-8 production through the P2Y6 signaling pathway. Blood 2006;107:2936-2942.

41. Shiba H, Mouri Y, Komatsuzawa H, Ouhara K, Takeda K, Sugai M, Kinane DF, Kurihara H. Macrophage inflammatory protein-3alpha and beta-defensin-2 stimulate dentin sialophosphoprotein gene expression in human pulp cells. Biochem Biophys Res Commun 2003;306:867-871.

42. Midorikawa K, Ouhara K, Komatsuzawa H, Kawai T, Yamada S, Fujiwara T, Yamazaki K, Sayama K, Taubman MA, Kurihara H, Hashimoto K, Sugai M. Staphylococcus aureus susceptibility to innate antimicrobial peptides, beta-defensins and CAP18, expressed by human keratinocytes. Infect Immun 2003;71:3730-3739.

43. Matsushita K, Motani R, Sakuta T, Yamaguchi N, Koga T, Matsuo K, Nagaoka S, Abeyama K, Maruyama I, Torii M. The role of vascular

endothelial growth factor in human dental pulp cells: induction of chemotaxis, proliferation, and differentiation and activation of the AP-1-dependent signaling pathway. J Dent Res 2000;79:1596-1603.

44. Kittaka M, Shiba H, Kajiya M, Ouhara K, Takeda K, Kanbara K, Fujita T, Kawaguchi H, Komatsuzawa H, Kurihara H. Antimicrobial peptide LL37 promotes vascular endothelial growth factor-A expression in human periodontal ligament cells. J Periodontal Res 2013;48:228-234.

45. De Yang, Chen Q, Schmidt AP, Anderson GM, Wang JM, Wooters J, Oppenheim JJ, Chertov O. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. J Exp Med 2000;192:1069-1074.

46. Elssner A, Duncan M, Gavrilin M, Wewers MD. A novel P2X7 receptor activator, the human cathelicidin-derived peptide LL37, induces IL-1 beta processing and release. J Immunol 2004;172:4987-4994.

47. Tokumaru S, Sayama K, Shirakata Y, Komatsuzawa H, Ouhara K, Hanakawa Y, Yahata Y, Dai X, Tohyama M, Nagai H, Yang L, Higashiyama S, Yoshimura A, Sugai M, Hashimoto K. Induction of keratinocyte migration via transactivation of the epidermal growth factor receptor by the antimicrobial peptide LL-37. J Immunol 2005;175:4662-4668.

48. Girnita A, Zheng H, Grönberg A, Girnita L, Ståhle M. Identification of the cathelicidin peptide LL-37 as agonist for the type I insulin-like growth factor receptor. Oncogene 2012;31:352-365.

49. Chen X, Niyonsaba F, Ushio H, Nagaoka I, Ikeda S, Okumura K, Ogawa H. Human cathelicidin LL-37 increases vascular permeability in the skin via mast cell activation, and phosphorylates MAP kinases p38 and ERK in mast cells. J Dermatol Sci 2006;43:63-66.

50. Birkedal-Hansen H, Moore WG, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A, Engler JA. Matrix metalloproteinases: a review. Crit Rev Oral Biol Med 1993;4:197-250.

51. Sekhon BS. Matrix metalloproteinases – an overview. Res Rep Biol 2010;1:1-20.

52. Chakraborti S, Mandal M, Das S, Mandal A, Chakraborti T. Regulation of matrix metalloproteinases: an overview. Mol Cell Biochem 2003;253:269-285.

53. Ra HJ, Parks WC. Control of matrix metalloproteinase catalytic activity. Matrix Biol 2007;26:587-596.

54. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol 2001;17:463-516.

55. Kähäri VM, Saarialho-Kere U. Matrix metalloproteinases and their inhibitors in tumor growth and invasion. Ann Med 1999;31:34-45.

56. Ravanti L, Kähäri VM. Matrix metalloproteinases in wound repair (review). Int J Mol Med 2000;6:391-407.

57. Palosaari H, Pennington CJ, Larmas M, Edwards DR, Tjäderhane L, Salo T. Expression profile of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs in mature human odontoblasts and pulp tissue. Eur J Oral Sci 2003;111:117-127.

58. Sulkala M, Pääkkönen V, Larmas M, Salo T, Tjäderhane L. Matrix metalloproteinase-13 (MMP-13, collagenase-3) is highly expressed in human tooth pulp. Connect Tissue Res 2004;45:231-237.

59. Suri L, Damoulis PD, Le T, Gagari E. Expression of MMP-13 (collagenase-3) in long-term cultures of human dental pulp cells. Arch Oral Biol 2008;53:791-799.

60. Morand MA, Schilder H, Blondin J, Stone PJ, Franzblau C. Collagenolytic and elastinolytic activities from diseased human dental pulps. J Endod 1981;7:156-160.

61. Shin SJ, Lee JI, Baek SH, Lim SS. Tissue levels of matrix metalloproteinases in pulps and periapical lesions. J Endod 2002;28:313-315.

62. Gusman H, Santana RB, Zehnder M. Matrix metalloproteinase levels and gelatinolytic activity in clinically healthy and inflamed human dental pulps. Eur J Oral Sci 2002;110:353-357.

63. O'Boskey FJ Jr, Panagakos FS. Cytokines stimulate matrix metalloproteinase production by human pulp cells during long-term culture. J Endod 1998;24:7-10.

64. Tamura M, Nagaoka S, Kawagoe M. Interleukin-1 alpha stimulates interstitial collagenase gene expression in human dental pulp fibroblast. J Endod 1996;22:240-243.

65. Wisithphrom K, Murray PE, Windsor LJ. Interleukin-1 alpha alters the expression of matrix metalloproteinases and collagen degradation by pulp fibroblasts. J Endod 2006;32:186-192.

66. Chang YC, Lai CC, Yang SF, Chan Y, Hsieh YS. Stimulation of matrix metalloproteinases by black-pigmented *Bacteroides* in human pulp and periodontal ligament cell cultures. J Endod 2002;28:90-93.

67. McDonald C, Inohara N, Nuñez G. Peptidoglycan signaling in innate immunity and inflammatory disease. J Biol Chem 2005;280:20177-20180.

68. Wong CK, Cheung PF, Ip WK, Lam CW. Intracellular signaling mechanisms regulating toll-like receptor-mediated activation of eosinophils. Am J Respir Cell Mol Biol 2007;37:85-96.

69. Chen BC, Liao CC, Hsu MJ, Liao YT, Lin CC, Sheu JR, Lin CH. Peptidoglycan-induced Il-6 production in RAW 264.7 macrophages is mediated by cyclooxygenase-2, $PGE_2$/$PGE_4$ receptors, protein kinase A, IκB kinase, and NF-κB. J Immunol 2006;177:681-693.

70. Kumar A, Zhang J, Yu FS. Innate immune response of corneal epithelial cells to Staphylococcus aureus infection: role of peptidoglycan in stimulating proinflammatory cytokine secretion. Invest Ophthalmol Vis Sci 2004;45:3513-3522.

71. Shiba H, Tsuda H, Kajiya M, Fujita T, Takeda K, Hino T, Kawaguchi H, Kurihara H. Neodymium-doped yttrium-aluminium-garnet laser irradiation abolishes the increase in interleukin-6 levels caused by peptidoglycan through the p38 mitogen-activated protein kinase pathway in human pulp cells. J Endod 2009;35:373-376.

72. Nagaoka I, Hirota S, Niyonsaba F, Hirata M, Adachi Y, Tamura H, Heumann D. Cathelicidin family of antibacterial peptides CAP18 and CAP11 inhibit the expression of TNF-alpha by blocking the binding of LPS to CD14(+) cells. J Immunol 2001;167:3329-3338.

73. Mookherjee N, Brown KL, Bowdish DM, Doria S, Falsafi R, Hokamp K, Roche FM, Mu R, Doho GH, Pistolic J, Powers JP, Bryan J, Brinkman FS, Hancock RE. Modulation of the TLR-mediated inflammatory response by the endogenous human host defense peptide LL-37. J Immunol 2006;176:2455-2464.

74. Kandler K, Shaykhiev R, Kleemann P, Klescz F, Lohoff M, Vogelmeier C, Bals R. The anti-microbial peptide LL-37 inhibits the activation of dendritic cells by TLR ligands. Int Immunol 2006; 18:1729-1736.

75. Shiba H, Takeda K, Ouhara H, Kajiya M, Fujita T, Mizuno N, Hino T, Kawaguchi H, Kurihara H. Effects of LL37 on expression of inflammatory cytokines in human pulp cells. J Jpn Endod Assoc 2008;29:147-154.

76. van Amerongen JP, Lemmens IG, Tonino GJ. The concentration, extractability, and characterization of collagen in human dental pulp. Arch Oral Biol 1983;28:339-345.

77. Rundhaug JE. Matrix metalloproteinases and angiogenesis. J Cell Mol Med 2005;9:267-285.

78. Kittaka M, Shiba H, Kajiya M, Fujita T, Iwata T, Khung R, Ouhara K, Takeda K, Fujita T, Komatsuzawa H, Kurihara H. The antimicrobial peptide LL37 promotes bone regeneration in a rat calvarial bone defect. Peptides 2013;46:136-142.

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