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Role of Vitamin D in Protecting the E-cadherin Junction of Gingival Keratinocytes

치은 각화상피세포에서의 E-cadherin 결합을 보호하는 비타민 D의 역할

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Role of Vitamin D in Protecting the E-cadherin Junction of Gingival Keratinocytes

by

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Abstract

Role of Vitamin D in Protecting the E-cadherin Junction of Gingival Keratinocytes

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Despite the well-known anti-inflammatory effects of vitamin D in periodontal health, its mechanism has not been fully elucidated. In the present study, the effect of vitamin D on strengthening E-cadherin junctions (ECJs) was explored in human gingival keratinocytes (HGKs). ECJs are the major type of intercellular junction within the junctional epithelium, where loose intercellular junctions develop and microbial invasion primarily occurs. HOK-16B cells, an immortalized normal

human gingival cell line, were used for the study. To mimic the inflammatory environment, cells were treated with tumor necrosis factor-alpha (TNF-α). Matrix metalloproteinases (MMPs) in the culture medium were assessed by an MMP antibody microarray and gelatin zymography. The expression of various molecules was investigated using western blotting. The extent of ECJ development was evaluated by comparing the average relative extent of the ECJs around the periphery of each cell after immunocytochemical E-cadherin staining. Vitamin D receptor (VDR) expression was examined via immunohistochemical analysis. TNF-α downregulated the development of the ECJs of the HGKs. Dissociation of the ECJs by TNF-α was accompanied by upregulation of MMP-9 production and was suppressed by a specific MMP-9 inhibitor. Exogenous MMP-9 decreased the development of ECJs. Vitamin D reduced the production of MMP-9 and attenuated the breakdown of ECJs in the HGKs treated with TNF-α. In addition, inhibition of nuclear factor kappa B (NF-κB) signaling attenuated the lowered development of ECJs in the HGKs treated with TNF-α. Vitamin D also downregulated TNF-α-induced nuclear factor kappa B (NF-κB) signaling in the HGKs. VDR was expressed in the gingival epithelium, including the junctional epithelium. These results suggest that vitamin D may avert TNF-α-induced downregulation of the development of

ECJs in HGKs by decreasing the production of MMP-9, which was upregulated by TNF-α. Vitamin D may reinforce ECJs by downregulating NF-κB signaling, which is upregulated by TNF-α. Vitamin D may protect the periodontium from bacterial invasion by strengthening the epithelial barrier.

Keywords: Cadherins, Keratinocytes, Matrix metalloproteinase 9, NF-kappa B, Tumor necrosis factor-alpha, Vitamin D

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1. INTRODUCTION

1-1. Junctional epithelium in periodontal disease

Gingival epithelium consists of three parts: oral gingival epithelium, sulcular epithelium, and junctional epithelium (JE). JE (Figure 1) consists of non-keratinized epithelial cells attached to the soft connective tissue of the lamina propria as well as the hard enamel surface of the tooth. Unlike other oral epithelial tissues, it is a specialized gingival epithelium with two basal laminae that seals the gap between the internal basal lamina and the gingival connective tissue (external basal lamina) to function as a barrier against the invasion of bacteria or bacterial toxins [1,2]. Humid, warm, and nutrient-rich environment of the oral cavity is perfect for the microorganisms to thrive. These microorganisms attach to the glycoprotein layer on the solid and non-shedding surfaces (tooth surfaces) to form biofilms. Since the biofilms form quickly on the exposed tooth surfaces, the tissues surrounding the biofilms are constantly exposed to periodontal pathogens. Therefore, specialized structural and functional roles of the junctional epithelium are required to control these risk factors [3,4]. Epithelial cells usually function as a barrier against invasion by pathogens. The cells are interconnected by tight junctions, adherens junctions,

desmosomes, and gap junctions. However, junctional epithelium cells in healthy periodontal tissues are linked by desmosomes or gap junctions, resulting in relatively sporadic, loose intercellular junctions [5]. In addition, since there is no keratinized epithelial cell layer that can function as a physical barrier, the junctional epithelium is more vulnerable to bacterial invasion, causing bacteria or bacterial toxins to spread over a large cell area. As a result, inflammation can progress quickly, leading to bone loss or loss of connective tissue attachment to the tooth [6,7]. As periodontal disease progresses, tissues and bone are destroyed, leading to pocket formation around the tooth. Over time, the pocket deepens, creating a larger space for bacteria to live [8]. As bacteria accumulate around the tooth and under the gingival tissue, more bacteria gather in the periodontal pocket, giving rise to greater bone and tissue loss. Therefore, the role of JE as a barrier and its contribution to preserving gingival and periodontal health is of great importance [9].

1-2. E-cadherin

Intercellular junctions between the epithelial cells include tight junctions, adherens junctions, desmosomes, and gap junctions. The adherens junctions organized by E-

cadherin play a major role in the structure of JE. E-cadherin, found on the surface of epithelial cells, is a single-pass transmembrane protein involved in Ca2+ dependent cell adhesion (Figure 2). E-cadherin consists of two domains. One side is bound to each other by the E-cadherin domains between the adjacent cells. On the other side, p120-catenin and β-catenin bind to the cytoplasmic domain and α-catenin, which is bound to the F-actin bundle, binds to β-catenin. E-cadherin junction is a protein complex that is continuously remodeled via assembling and disassembling. It plays a crucial role in cell behavior, tissue formation and suppression of cancer [7,10]. Loss of e-cadherin is a well-known and important mechanism for promoting progression of diseases such as cancer and tissue fibrosis. Particularly, the loss of E-cadherin is closely related to epithelial-mesenchymal transition (EMT). Downregulation of epithelial markers such as E-cadherin and upregulation of mesenchymal markers such as vimentin are the main features of EMT. The loss of cell-to-cell adhesion and the loss of cell junctions mediated by E-cadherin binding occur frequently during tumor metastasis, which is primarily isolated from the tumor and invades the surrounding tissues, moving to distant sites [11,12]. EMT is regulated by various growth factors and cytokines. TGF-β is considered a key factor for inducing EMT in several cell lines. In some cell lines, cytokines such as TNF-α

and IL-6 are also known to induce EMT [13,14,15]. E-cadherin shows normal expression in healthy epithelium, but is markedly reduced in junctional epithelium affected by a disease. It performs an important barrier function to protect against external bacteria and toxins.

1-3. Matrix metalloproteinase & Tissue inhibitor of metalloproteinases

The matrix metalloproteinase (MMP) family is known to be involved in tumor cell invasion and metastasis by mediating the degradation of extracellular matrix components. According to substrate preference, MMPs are classified into type IV collagenases (MMP-2,9), stromelysins (MMP-3,10), and interstitial collagenases (MMP-1,8). Type IV collagenases are divided into gelatinase A (MMP-2, 72KDa) and gelatinase B (MMP-9, 92kDa). [16] In vivo, MMP-2 is produced by fibroblasts, endothelial cells, and macrophages and degrades Type IV and type V collagen. MMP-9 is produced by both normal and cancer cells and is closely related to the malignancy of the tumor cells [17].

Tissue inhibitors of metalloproteinases (TIMPs) are inhibitors of MMPs that break down the extracellular matrix. Since only tumor cells develop invasive growth

despite the release of MMPs from tumor cells as well as normal cells, it can be inferred that MMP activity in tumors is regulated by some factor and active inhibitors such as TIMPs play an important role [16,18]. Four types of TIMPs are known (TIMP 1,2,3, and 4), of which TIMP-1 binds to gelatinase B proenzyme to inhibit the action of MMP-9 and TIMP-2 binds to gelatinase to inhibit the action of MMP-2 [19].

1-4. MMP-9

MMP-9 (92kDa) is a zinc-dependent endopeptidase involved in the degradation of extracellular matrix. It belongs to the gelatinase subgroup of the MMP family. MMPs are regulated through gene transcription, protein translation, pro-MMP activation, and endogenous inhibition. Activated MMP-9 is involved in embryonic development and reproduction, cell migration, neutrophil action, angiogenesis, wound repair, and cancer progression [20]. MMP-9 is synthesized as a pre-proenzyme of 707 amino acid residues including 19 amino acid signal peptides and is secreted in the form of inactive pro-MMP. Usually, MMP-9 degrades gelatin and collagen, the main components of the basement membrane. During the inflammatory response, the

migration and infiltration of inflammatory cells is promoted by the MMPs. During tissue development and remodeling, MMPs and TIMPs are secreted simultaneously. Breakdown of the balance between MMP-9 and TIMP-1 results in diseases such as rheumatoid arthritis, osteoarthritis, lung disease, kidney disease, fibrosis, and tumor invasion [21]. Recent reports have shown increased expression of MMPs in almost all human diseases with inflammation. In vitro studies and studies in mouse models have reported that MMP-9 works extensively in defense, injury, and inflammation [22,23]. In addition, MMP-9 is associated with periodontal inflammation. During the inflammatory response in periodontal connective tissue, cytokines such as IL-1β, IL-6, and TNF-α are released from JE, connective tissue fibroblasts, and macrophages. MMP-8 and MMP-9 are produced by the polymorphonuclear leukocytes (PMNs) and osteoclasts, leading to connective tissue and alveolar bone resorption. PMNs play an important role in regulating MMP-9 in periodontitis. Periodontal tissue destruction is associated with high levels of active MMP-9 in gingival crevicular fluid [24].

1-5. NF-κB signaling as pro-inflammation signaling

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a transcription factor that regulates adaptive immune response and functions as a pivotal mediator of inflammatory responses. NF-κB is involved in inflammasome regulation by inducing various pro-inflammatory gene expressions including encoding of cytokines and chemokines [25]. The NF-κB family consists of five members including NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel. In the absence of stimuli, NF-κB is segregated in the cytoplasm and must be translocated to the nucleus to perform its function. In mammals, the most important regulator of NF-κB is nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-alpha (IκBα). When IκBα binds to p50 and p65, it remains a heterodimer in the cytoplasm and prevents transcriptional activation of the NF-κB target gene [26]. NF-κB activation has 2 major pathways, namely canonical and non-canonical. Although there are differences in signal mechanisms, both are important for regulating immune and inflammatory responses [25,27]. The canonical NF-κB pathway (Figure 3) responds to a variety of stimuli including ligands of various cytokine receptors, TNF receptor (TNFR), T-cell receptor (TCR), and B-cell receptor. The first mechanism of NF-κB activation is degradation of IκBα by the IκB kinase

(IKK) complex. IKK consists of a regulatory subunit called IKKγ (NEMO), which is an essential regulator of IKKα, IKKβ subunits, and NF-κB. IKK phosphorylates IκBα of two N-terminal serines, resulting in ubiquitin dependent IκBα degradation in proteasomes. As a result, p50/RelA and p50/c-Rel dimers enter the nucleus and act as transcription factors [25,28]. The non-canonical NF-κB pathway selectively responds to specific stimuli groups including ligands from TNFR superfamily members such as LTβR, BAFFR, CD40, and RANK. Canonical pathways are involved in almost all aspects of the immune response, while non-canonical pathways regulate the specific functions of the adaptive immune system [25].

1-6. Vitamin D as an anti-inflammatory agent

Calcitriol (1, 25-dihydroxycholecalciferol), the active form of vitamin D, is commonly produced in the kidneys. Besides calcium and phosphorus regulatory functions of vitamin D for mineral homeostasis, there is increasing interest in the role of vitamin D in controlling the progression of inflammatory diseases. Vitamin D deficiency results in diseases such as cancer, cardiovascular disease, osteoarthritis, sarcopenia, infection, and rejection of transplants and autoimmune diseases such as

diabetes, multiple sclerosis, and inflammatory bowel disease [29,30,31]. Expression of many inflammation-related genes is regulated through vitamin D receptor (VDR) in a variety of cells including immune cells such as macrophages, dendritic cells, helper T cells, and B cells. Vitamin D shows anti-inflammatory effects through activated B cells (NF-κB) by regulating the biosynthesis of pro-inflammatory molecules or by affecting the cytokine production and inflammatory responses [29]. Studies have reported the effects of vitamin D on inflammatory cells and processes. In vitro, vitamin D promotes differentiation of macrophages to prevent the release of inflammatory cytokines and reduces antigen presentation by inhibiting cell surface expression of MHC-II molecules [30,32]. Vitamin D inhibits the proliferation and stimulatory abilities of T cells and monocytes. It downregulates the pro-inflammatory cytokines including TNF-α, IL-6, IL-1, and IL-8 and upregulates anti-inflammatory cytokines. The absence of VDR and increased NF-κB activity, which plays a major role in immune regulation, have been reported to be related to various inflammatory conditions. Moreover, studies have shown that vitamin D blocks NF-κB translocation and weakens its activity, demonstrating its anti-inflammatory effect [33].

1-7. Vitamin D in periodontal health

Inflammation in the oral cavity includes gingivitis caused by inflammation of the gingival epithelium and connective tissue and periodontitis induced by gingival bacterial plaque, which leads to gradual destruction and subsequent loss of periodontal connective tissue and bone. Host response to microbial antigens in gingivitis and periodontitis involves the action of adaptive immunity as well as innate immunity, both of which are known to be regulated by vitamin D [34,35]. Molecules that cause periodontal destruction include virulence factors (lipopolysaccharides, bacterial enzymes, noxious products, fimbriae, and bacterial and extracellular deoxyribonucleic acid) and cytokines derived from the host immune inflammatory responses [36,37]. In a previous study, periodontal attachment loss and serum vitamin D levels were found to be inversely related to each other regardless of smoking and diabetes [38]. Another study reported the in vitro biological action of intercellular VDR and vitamin D, explaining the association between the active form of vitamin D and periodontal inflammation and tissue destruction [39]. Using cross-section, vitamin D deficiency has been shown to increase the risk of chronic periodontitis and supplementation with vitamin D alone is effective in maintaining periodontal health, increasing mineral density in the jaw,

and inhibiting bone resorption [40]. Indeed, a study regarding the association between total vitamin D intake and periodontal health in 562 elderly participants with a mean age of 62.9 years found that those with more than 800 IU of daily total vitamin D intake had lower levels of severe periodontal disease than those with less than 400 IU daily intake [33,41]. Vitamin D is also known to combat bacterial erosion of gingiva by increasing the production of antimicrobial peptides in gingival epithelial cells [42,43].

1-8. Vitamin D protecting the gingival epithelium including JE

Despite the anti-inflammatory potential of vitamin D in periodontal health, studies on the mechanisms associated with the anti-inflammatory effects of vitamin D in periodontal health are limited. Vitamin D has been reported to enhance the production of anti-microbial peptides in gingival epithelial cells that encounter bacteria invading the gingiva and to regulate the production of pro-inflammatory cytokines by gingival epithelial cells and periodontal ligament cells infected with oral bacteria [42,43]. In this study, the effect of vitamin D on the development of intercellular junctions, which are the first line of defense as an epithelial barrier against attack by microorganisms was investigated [3]. Maintaining intact intercellular junctions in the epithelium is imperative because they may impede the

invasion of microorganisms or toxins through the epithelial barrier [2]. In particular, slight dissociation of the intercellular junction in the junctional epithelium (JE), where intercellular junctions are characteristically sporadic and loose, may cause the epithelial barrier of the JE to deteriorate [5]. Breakdown of intercellular junctions in the JE may promote pathologic desquamation of the keratinocytes facing the gingival sulcus in the upper JE and deepen the gingival sulcus at the initial stages of inflammation. In addition, this breakdown may be associated with denudation of the narrow pocket epithelium, exposing the connective tissue under the pocket epithelium at the later stage of inflammation [8].

2. PURPOSE OF THE STUDY

E-cadherin is critical for maintaining the structural integrity of the oral epithelium, including the JE [3,7]. In the present study, E-cadherin intercellular junctions as the target of tumor necrosis factor-alpha (TNF-α) was evaluated, because E-cadherin forms the major intercellular junctions of the JE [7]. Furthermore, E-cadherin is regarded as a key molecule in the development of desmosomes, as well as adherens junctions [4]. Expression of E-cadherin is mostly reduced in the inflammatory JE [5,44] and dissociation of E-cadherin increases the permeability of the intercellular

junctions in HGKs [14]. Herein, the effect of vitamin D on the disruption of E-cadherin junctions (ECJs), which are associated with the epithelial barrier, was explored in gingival epithelial cells that were challenged by the pro-inflammatory cytokine TNF-α. It is well-known that TNF-α hampers the epithelial barrier in gingival keratinocytes [14,15]. TNF-α is produced at high levels by gingival epithelial cells infected with oral bacteria such as *Porphyromonas gingivalis* [42]. Gingival keratinocytes that secrete TNF-α might affect themselves in an autocrine fashion. In particular, gingival keratinocytes in the JE, where the intercellular space is wide enough [5] to accommodate macromolecules due to the incomplete intercellular junctions, may be directly confronted by self-secreted TNF-α. TNF-α may be derived from inflammatory cells in the connective tissue in the later stages of gingival or periodontal inflammation.

This study was performed to determine whether TNF-α dissociated the ECJs of human gingival keratinocytes (HGKs) via upregulation of MMP-9 production by HGKs and whether vitamin D reduced the breakdown of the E-cadherin intercellular junctions of HGKs by downregulating the production of MMP-9 by HGKs.

3. MATERIALS & METHODS

3-1. HGK cultures

The HOK-16B cell line, which was immortalized from healthy human retromolar gingival tissues, was used in this study [18]. The HOK-16B cell line was a gift from Dr. N. H. Park (School of Dentistry, University of California, Los Angeles, CA, USA). The HGKs (HOK-16B cells) were cultured in supplemented keratinocyte growth medium (Lonza, Basel, Switzerland) in 5% CO2 at 37°C. The medium supplement contained bovine pituitary extract, recombinant human insulin, hydrocortisone, recombinant human epidermal growth factor, gentamicin sulfate-amphotericin (GA-1000), and 1% penicillin. The following reagents were used in the culture of HOK-16B cells; rh-MMP-9 (MMP-9) from Sino Biological (Wayne, PA, USA), rh-tissue inhibitor of metalloproteinase-1 (TIMP-1) from Calbiochem (San Diego, CA, USA), rh-TNF-α from Peprotech (Rocky Hill, NJ, USA), and 1α,25-dihyhroxyvitamin D3 (vitamin D) and Bay 11-7082 (a pharmacological inhibitor of NF-κB signaling) from Sigma-Aldrich (St. Louis, MO, USA). The MMP-9 specific inhibitor was purchased from Abcam (Cambridge, UK). Treatment doses and times are shown in the figure legends.

3-2. Immunoblotting

Immunoblotting was performed according to a standard protocol. Briefly, the cells were lysed with a RIPA lysis buffer (150 mM NaCl, 0.5% deoxycholic acid, 50 mM Tris-HCl [pH 7.5], 1% NP-40, and 0.1% sodium dodecyl sulfate [SDS]) containing a protease inhibitor mixture comprising 1 mM Na_3VO_4, 10 mM NaF, and 1 mM PMSF protease inhibitor (Boehringer Mannheim, Indianapolis, IN, USA). Cell lysates that were boiled in sample buffer were run through SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes. Nonspecific reactions of the membranes were blocked with 5% skim milk, followed by incubation with primary antibodies overnight at 4°C. Antibodies for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), NF-κB p65, p-NF-κB p65, and E-cadherin were purchased from Cell Signaling Technology (Danvers, MA, USA). The incubated membranes were reacted with secondary antibodies in 5% skim milk at room temperature for 1 hour. Horseradish peroxidase (HRP)-linked immunoglobulin G (IgG) for various animals was purchased from Cell Signaling Technology. The blots were developed using an enhanced chemiluminescent HRP substrate (Thermo-Fisher Scientific/Invitrogen, Waltham, MA, USA).

3-3. Antibody microarray analysis of MMPs

Antibody microarray analysis of MMPs was conducted following the manufacturer's instructions. Briefly, antibody microarray chips for the analysis of MMPs (Abcam) were blocked with blocking buffer for 30 minutes. Then, the chips were incubated in conditioned media at 4°C overnight. Thoroughly washed incubated chips were reacted with biotin-conjugated detector antibody cocktail for 2 hours. The chips were next incubated with HRP-conjugated streptavidin for 2 hours. Then, the reaction products were visualized by reacting HRP with a chemiluminescent substrate. The relative amount of MMP-9 was compared based on the intensity of MMP-9 bands obtained using ImageJ (National Institutes of Health, Bethesda, MD, USA). Specifically, the amount of MMP-9 was calculated by dividing the intensity of MMP-9 by the average intensity of 3 positive control references included in each blot as an internal control.

3-4. Zymogram analysis

Proteins in the conditioned medium were separated by SDS-PAGE using 7.5% acrylamide gel containing gelatin (4 mg/mL). The zymogram buffer kit was

purchased from Koma Biotech (Seoul, Korea). After washing the gel to remove SDS with zymogram renaturing buffer, the gel was incubated to induce an enzyme-substrate reaction in the zymogram development buffer at 37°C for 48 hours. Then, the incubated gel was stained with Coomassie brilliant blue G-250 (Bio-Rad, Hercules, CA, USA) to stain the unreacted gelatin. The relative activity of MMP-9 was compared based on the intensity of the MMP-9 bands obtained using ImageJ.

3-5. Immunocytochemistry and measurement of ECJs

Immunocytochemical staining followed a standard protocol. Briefly, cells were fixed with 4% paraformaldehyde for 10 minutes and permeabilized with 0.2% Triton X-100 for 30 minutes. The permeabilized cells were incubated with primary antibodies for 2 hours at room temperature. E-cadherin was obtained from Cell Signal Technology as a primary antibody. Then, cells were treated with Cy3-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch Lab, West Grove, PA, USA) for 1 hour. F-actin and nuclei were stained with FITC-phalloidin (Sigma-Aldrich) or 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich), respectively. The stained cells were photographed with serial Z-sections using an

LSM 700 confocal laser-scanning microscope (Zeiss, Oberkochen, Germany). In addition, images of the expression of E-cadherin and F-actin were merged to examine the colocalization of E-cadherin and F-actin. Dissociation of the ECJs began with separation of the E-cadherin band from the F-actin band. Thus, whether E-cadherin and F-actin were colocalized in the merged images of both E-cadherin and F-actin served as a quick reference to discern the dissociation of ECJs. The development of intercellular junctions in HGKs was evaluated by measuring the length of ECJs that developed around the perimeter of the cells. The extent of ECJ development was designated by averaging the relative development of ECJs from 20–30 cells that were randomly chosen from multiple experiments [45]. The relative extent of ECJ development per cell was obtained by dividing the length of the E-cadherin expressed at the cell junctions by the total perimeter of the cell; these values were obtained by tracing using ImageJ.

3-6. Immunocytochemistry for NF-κB translocation to nucleus

After permeabilization with 0.2% Triton X-100 for 30 minutes, the cells were incubated with NF-κB p65 (Biolegend, San Diego, CA, USA) as a primary antibody.

NF-κB p65 primary antibody was treated overnight at 4°C. Subsequently, the cells were treated with Cy3-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch Lab, West Grove, PA, USA), FITC-phalloidin (Sigma-Aldrich, St. Louis, MO, USA), and 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA). From the step involving the secondary antibody to the last step, the procedure was performed in a way similar to the E-cadherin staining sequence and treatment time. Comparisons were performed among control, TNF-α treatment group, and TNF-α with vitamin D treatment group using NF-κB translocated into the nucleus, which was detected by confocal microscopy images.

3-7. Immunohistochemistry of vitamin D receptor (VDR)

Immunohistochemical analysis involving animals followed protocols approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-180207-3). Six-week-old Sprague Dawley rats were sacrificed by CO2 asphyxiation. Mandibular molars, including the surrounding tissues, were fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4) at 4°C overnight. Decalcified tissues in a solution of 10% ethylenediaminetetraacetic acid (pH 7.4) were embedded in

paraffin, and then processed for the immunohistochemical examinations. The deparaffinized sections were treated to retrieve antigens in a solution of citrate buffer (pH 6.0) at 60°C overnight. Expression of VDR was detected using an ABC kit (Vector Labs, Burlingame, CA, USA) and a DAB kit containing nickel chloride to enhance the contrast of the immunoreactivity (Vector Labs) after treating tissues with rabbit anti-VDR (1:50, Santa Cruz, Santa Cruz, CA, USA) as the primary antibody and a biotin-labeled goat anti-mouse IgG (1:200, Vector Labs) as the secondary antibody. The negative control slides were treated similarly, except that the reaction with the primary antibody was omitted.

3-8. Statistical analysis

All data on the relative extent of ECJ development per cell are presented as the mean±standard deviation of the mean. Statistical analyses for the comparison of the means were performed using MedCalc for Windows (version 18.11, MedCalc software, Ostend, Belgium). After normality analysis by the Kolmogorov-Smirnov test, depending on the normality of the data distribution, the nonparametric Kruskal-Wallis test and post hoc multiple comparison with the Dunn test or parametric 1-way

analysis of variance and post hoc multiple comparison with the Tukey-Kramer test was performed. The significance level was set as $P<0.05$. The results of non-quantitative immunoblotting analyses, microarray analyses, zymogram analyses, and immunohistochemical observations are presented as a representative example of the 2 or 3 experiments, respectively, all of which showed consistent results from independent cultures.

4. RESULTS

4-1. ECJs with TNF-α & Vitamin D

ECJs were downregulated when the HGK cells were treated with TNF-α (Figure 4). Interestingly, the preformed ECJs were dissociated by treatment with TNF-α. The effects of vitamin D on the development of E-cadherin were examined to determine whether vitamin D averted the attenuated development of the ECJs in the HGKs in response to TNF-α. As expected, vitamin D nullified the deteriorating effect of TNF-α on the development of ECJs of gingival epithelial cells (Figure 5).

4-2. Disruption of ECJs by MMP-9

To find a candidate molecule that may mediate the vitamin D-induced strengthening of the intercellular junctions of the gingival keratinocytes in the inflammatory setting, HOK-16B cells were treated with TIMP-1. This was performed to examine whether MMPs were involved in the breakdown of the intercellular junction by TNF-α as MMPs have been reported to destroy various epithelial intercellular junctions, including ECJs [46-50]. As expected, TIMP-1 reversed the destruction of ECJs by TNF-α (Figure 6). Next, the type of MMPs mediating the destruction of ECJs by

TNF-α was explored through antibody microarray analysis of the conditioned culture medium. An antibody microarray analysis of MMPs showed that the secretion of MMP-9, not MMP-1, 2, 3, 8, 9, 10, or 13, was upregulated in the culture medium by TNF-α (Figure 7A). The upregulation of MMP-9 by TNF-α was further confirmed by gelatin zymogram analysis. TNF-α upregulated the activity of MMP-9 in the culture medium in a dose-dependent manner in the zymogram analysis (Figure 7B). In addition, treatment of the cells with a specific inhibitor for MMP-9 maintained the development of ECJs at the rate of the control despite treating cells with TNF-α (Figure 8). Next, since dissociation of ECJs by MMP-9 has not been reported in oral keratinocytes, including gingival keratinocytes, except for a few cases involving cancer cells, the direct effect of exogenous MMP-9 on the downregulation of ECJs was examined. MMP-9 directly reduced the development of ECJs (Figure 9). These results suggest that MMP-9 may be involved in the break-up of the epithelial barrier by TNF-α in gingival epithelial cells.

4-3. Reinforcement of ECJs by vitamin D

Next, it was investigated how vitamin D averted the dissociation of the ECJs by

TNF-a. Based on the antibody microarray analysis and zymogram analysis, vitamin D reduced the production of MMP-9 that was upregulated by TNF-α (Figure 10). Along with upregulating the development of ECJs, these results indicate that vitamin D may fortify ECJs against dissociation by TNF-α through downregulating the production of MMP-9, which is destructive to the ECJs. Next, target molecules of vitamin D were explored to further elucidate the mechanism through which vitamin D strengthened the development of ECJs against the deleterious effects of TNF-α by downregulating MMP-9 production. Since NF-κB is a well-known downstream actor in the signal transduction pathway of TNF-α stimulation in inflammatory reactions [25], NF-κB signaling was explored to determine whether this pro-inflammatory signaling pathway played a role in the destruction of ECJs and the upregulated production of MMP-9 by TNF-α in HGKs. Treatments of the HGKs with Bay 11-7082 along with TNF-α clearly averted the attenuated development of the ECJs (Figure 11) and the upregulated production of MMP-9 by TNF-α (Figure 12). The finding that the deleterious effects of TNF-α were reversed by Bay 11-7082 (Figures 11 and 12), as was observed for vitamin D (Figures 5 and 10), strongly indicates that vitamin D works through attenuating NF-κB signaling to protect the epithelial barrier and reduce the production of MMP-9 under the influence of TNF-α. Furthermore,

the downregulation of NF-κB signaling was directly demonstrated by immunoblotting analyses. As expected, vitamin D downregulated the NF-κB signaling that was upregulated by TNF-α (Figure 13A). These results indicate that vitamin D may reinforce ECJs by downregulating inflammatory NF-κB signaling, which is upregulated by TNF-α. In immunocytochemistry, strongly stained nuclei indicated an increase in TNF-α-induced NF-κB translocation to the nucleus when compared with the control group. On the other hand, in the group treated with TNF-α and vitamin D, the NF-κB translocation to the nucleus was significantly reduced. These results suggest that vitamin D might block TNF-α-induced NF-κB activation (Figure 13B). Next, VDR expression was examined in the gingival epithelium, since the presence of VDR in the gingival epithelium would be promising in terms of the clinical relevance of the present in vitro study. VDR was clearly expressed throughout the entire layer of the gingival epithelium, including the JE, sulcular epithelium, and oral gingival epithelium (Figure 14).

5. DISCUSSION

The present study shows, for the first time, that vitamin D attenuated the dissociation of the ECJs of HGKs in response to TNF-α. VDR was highly expressed in the gingival epithelium, including the JE. Preservation of stable ECJs in the gingival epithelium by vitamin D may fortify the epithelial barrier in pro-inflammatory conditions. The maintenance of epithelial barrier integrity via vitamin D may be a factor involved in the protection of the periodontium against bacterial invasion. Although no report has shown an upregulating effect of vitamin D on the development of ECJs in gingival keratinocytes, vitamin D has been indirectly suggested to be associated with the development of ECJs in gingival keratinocytes. Gingival keratinocytes from mice deficient in VDR developed fewer ECJs than those from wild-type mice in vitro [51]. The association of ECJs with vitamin D is also well-characterized in other cells. Vitamin D was found to enhance the expression of E-cadherin in bronchial epithelial cells [52]. Vitamin D upregulated ECJs to suppress the epithelial-mesenchymal transition in various cancer cells [53,54]. In addition to the effects of vitamin D on the development of ECJs, increased production of antimicrobial peptides [42,43] and decreased production of pro-inflammatory cytokines [42] from gingival keratinocytes infected by *P. gingivalis* were observed

when gingival keratinocytes were treated with vitamin D. Such fortification of the epithelial barrier depleted by TNF-α has also been reported in the gingival epithelium in response to substances other than vitamin D. Azithromycin, an antibiotic, and irsogladine maleate, a gastric mucosal protectant, promoted recovery of the epithelial barrier downregulated by TNF-α in HGKs [14,55]. In a recent report, estrogen was reported to upregulate the epithelial integrity of HGKs diminished by TNF-α [15]. Turning to other epithelial systems, azithromycin also maintained epithelial integrity in airway epithelial cells [56], and dexamethasone reversed the downregulation of E-cadherin expression in bronchial epithelial cells [57]. Vitamin D is believed to mitigate the destructive effects of TNF-α, which is known to be secreted from keratinocytes when infected by oral bacteria such as *P. gingivalis* [42]. Destruction of the epithelial barrier by TNF-α is a well-known phenomenon in various epithelial cells [13,57,58] including gingival keratinocytes [14,15]. Transepithelial electric resistance through the sheet of gingival keratinocytes was reduced by treatment with TNF-α [14,15]. However, the mechanism through which vitamin D downregulates the development of the intercellular junctions of gingival keratinocytes remains unclear. In the present study, for the first time, it was demonstrated that TNF-α dissociates ECJ by stimulating the production of MMP-9 in HGK. The dissociation

of ECJs by exogenous MMP-9 indicates that MMP-9 directly disassembles ECJs in HGKs. Although in the present study, dissociation of the E-cadherin intercellular junction by MMP-9 did not show a dramatic ability to separate the gingival epithelial cells to the extent that is observed in the epithelial-mesenchymal transition, even a low level of ECJ disassembly may significantly damage the intercellular junctions of the keratinocytes in the JE, where only partial and weak ECJs are developed. Breakdown of the weak epithelial barrier in the JE may allow the invasion of bacteria to proceed farther or promote desquamation of the upper cells, making the gingival sulcus deeper. As a mechanism by which vitamin D fortifies epithelial integrity, in the present study, it was demonstrated that MMP-9 responds to vitamin D. Vitamin D downregulated the MMP-9 production that was upregulated by TNF-α. Although downregulation of MMP-9 by vitamin D has not been reported in gingival keratinocytes, it has been documented in various cancer cells [48-50]. MMP-9, a zinc-metalloproteinase, degrades extracellular matrix components such as type IV collagen, type V collagen, and gelatin to promote angiogenesis and wound healing [60]. Interestingly, it also dissociates ECJs by causing the extracellular domain of E-cadherin receptors to be shed in ovarian carcinoma cells [46] and renal tubular epithelial cells [47]. At this moment, however, it is not clear whether MMP-9 also

cleaves the exo-domain of E-cadherin receptors to destroy ECJs in HGKs. Further study is required to elucidate how MMP-9 dissociates ECJs in HGKs. It is also noteworthy that HGKs secrete MMP-9, since MMP-9 is regarded as a specific marker representing periodontitis when detected in gingival crevicular fluid or saliva [60,61]. The production of MMP-9 by gingival keratinocytes suggests the possibility that epithelial MMP-9 may be an initial source of the MMP-9 found in the crevicular fluid or saliva before the later production of MMP-9 by inflammatory cells in the connective tissue. Gingival keratinocytes are known to secrete MMP-9 into the crevicular fluid; this process is triggered by TNF-α the expression of which is upregulated by infection with *P. gingivalis* [42]. In the present study, vitamin D treatment diminished NF-κB signaling, which was upregulated by TNF-α stimulation; furthermore, vitamin D also stimulated upregulation of ECJs and downregulation of MMP-9 production in the HGKs. NF-κB signaling is a prototypical pro-inflammatory signaling pathway [25] that is highly upregulated by TNF-α [62]. Thus, the idea that NF-κB signaling is a target of vitamin D signaling is not surprising. The finding that the downregulation of ECJs and the upregulation of MMP-9 production by TNF-α was averted by Bay 11-7082, a specific pharmacological NF-κB inhibitor, as was observed in response to vitamin D, further

supports the possibility that vitamin D may reduce the pro-inflammatory TNF-α signaling pathway through downregulating NF-κB signaling. In addition, the downregulation of p-NF-κB by vitamin D, as shown in immunoblotting analyses, further suggests that vitamin D suppresses NF-κB signaling by TNF-α, through which the ECJs were destroyed by means of upregulated MMP-9 expression, to protect the epithelial barrier. In oral keratinocytes, vitamin D has been reported to reduce lipopolysaccharide-induced inflammation by downregulating NF-κB signaling [63]. Furthermore, VDR expression in the JE, as observed in the present study, suggests the possibility that vitamin D may substantially regulate ECJs to reinforce the epithelial barrier in the JE. In skin keratinocytes, NF-κB has also been proposed as a target molecule for vitamin D in reducing inflammation [64]. NF-κB signaling may be a target for developing more potent vitamin D analogs to reinforce the integrity of the epithelial barrier against bacterial challenges. In pancreatic cancer cells, a vitamin D analog, MART-10 (19-nor-2α-[3-hydroxypropyl]-1α,25[OH]2D3), was found to be more potent than vitamin D in terms of both reduction of MMP-9 production and loss of E-cadherin. It is expected that various vitamin D analogs will be tested to find more efficient agents for strengthening the epithelial integrity of the gingival keratinocytes through mechanistically targeting NF-κB signaling.

6. CONCLUSION

The results of the present study suggest that vitamin D may avert TNF-α induced downregulation of the intercellular ECJs of HGKs, decreasing the production of MMP-9 by TNF-α. In addition, vitamin D may reinforce ECJs by downregulating NF-κB signaling, which is upregulated by TNF-α. Such maintenance of the epithelial barrier by vitamin D may be associated with a role of vitamin D in protecting the periodontium from oral bacteria. **Figure 15** summarizes the results of the present study.

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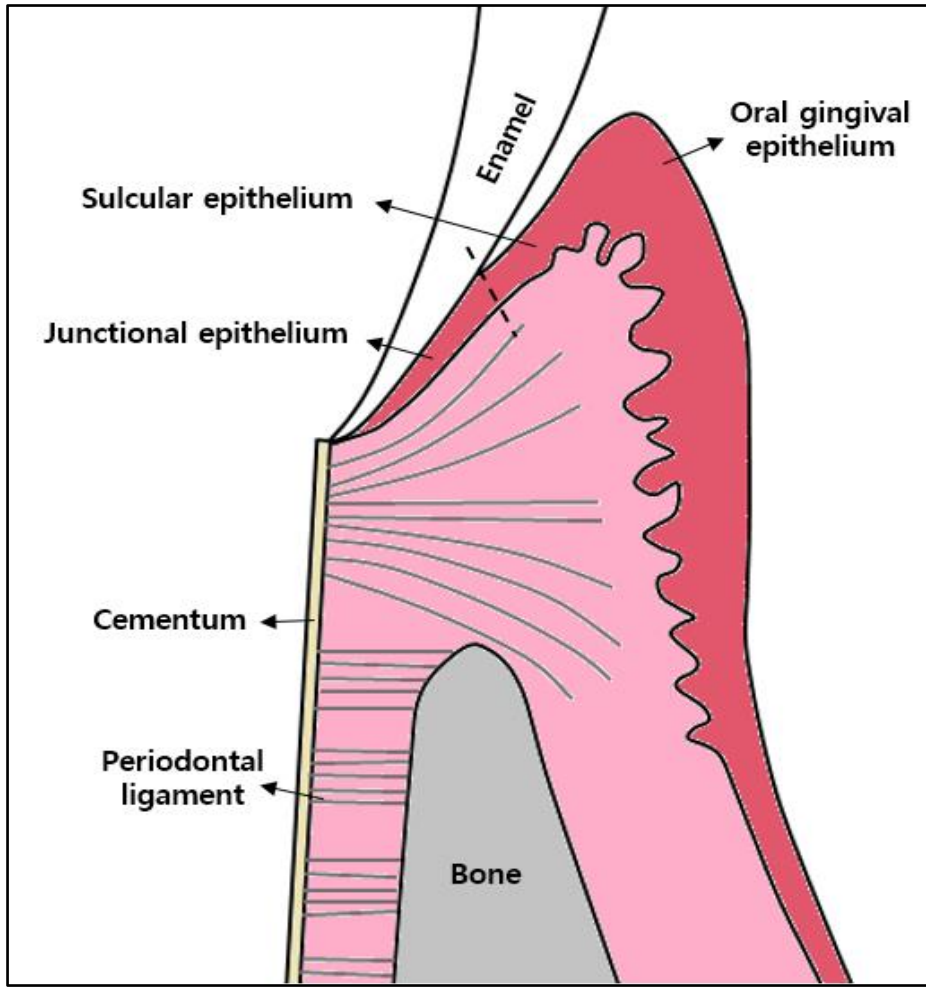


Figure 1. Junctional epithelium is an interface between tooth and gingiva. (Modified from [65])

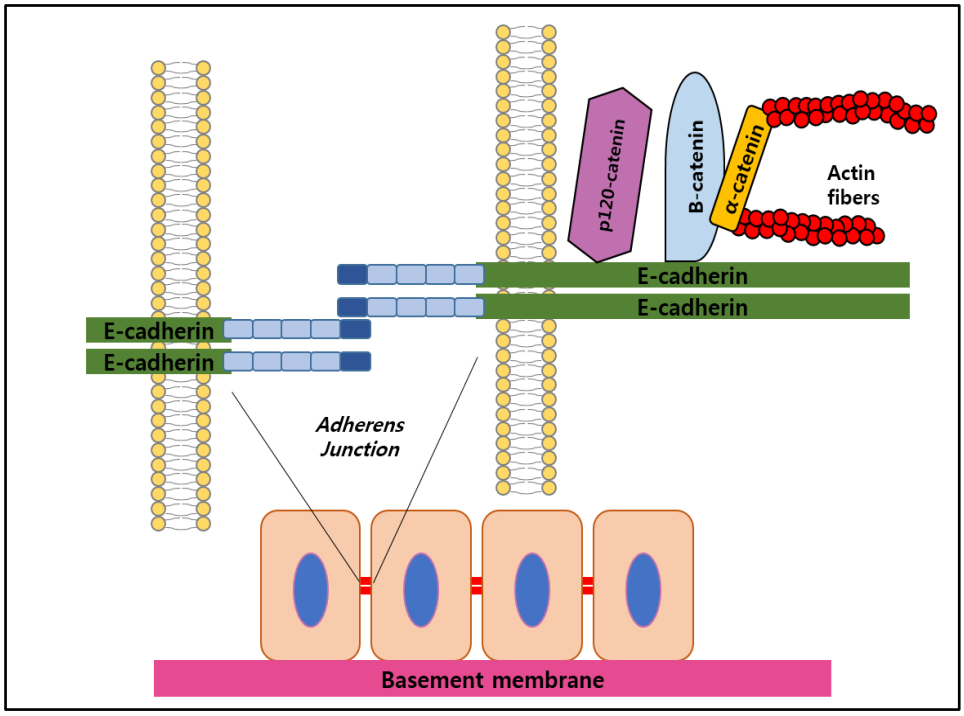


Figure 2. Molecular structure of E-cadherin junction. (Modified from [66])

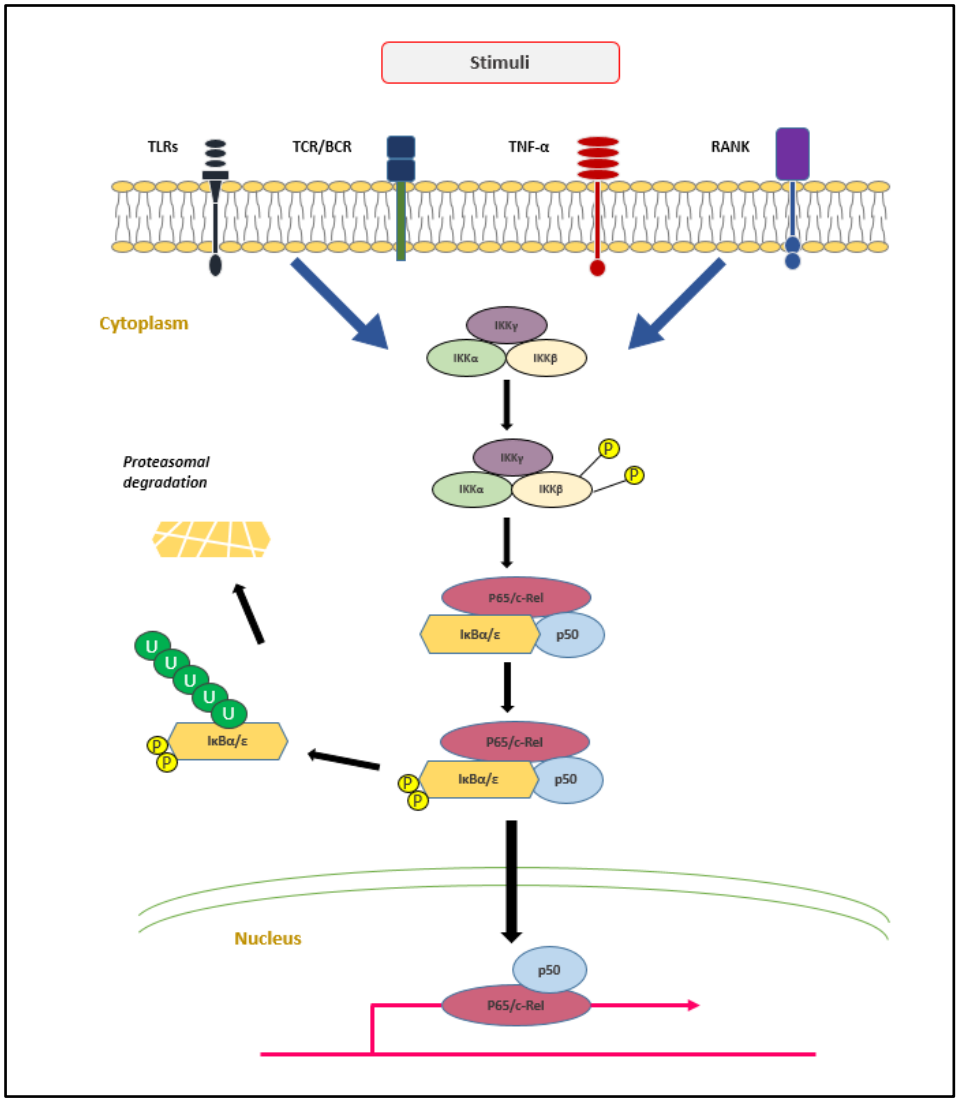


Figure 3. Diagram of canonical NF-κB pathway. (Modified from [67])

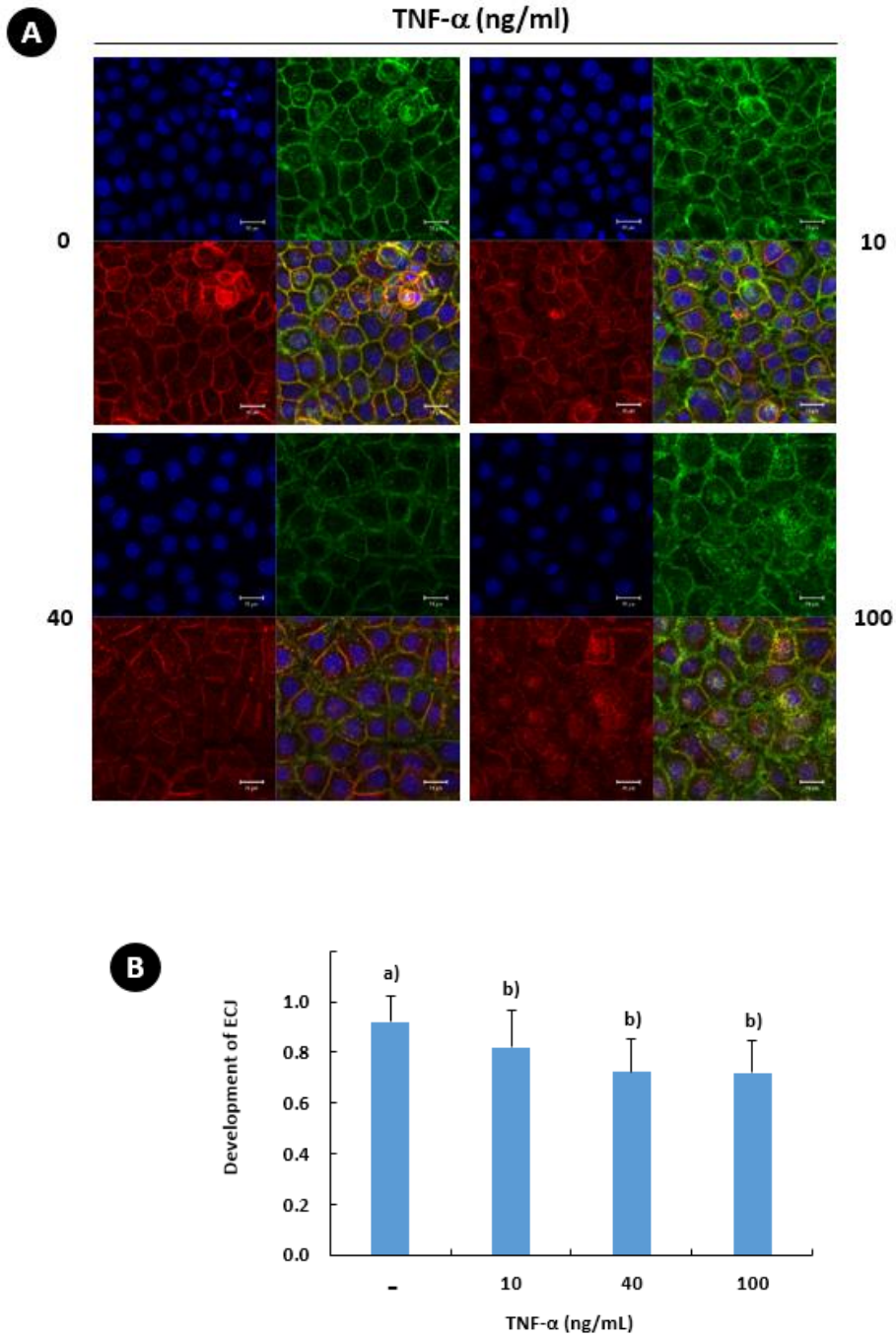
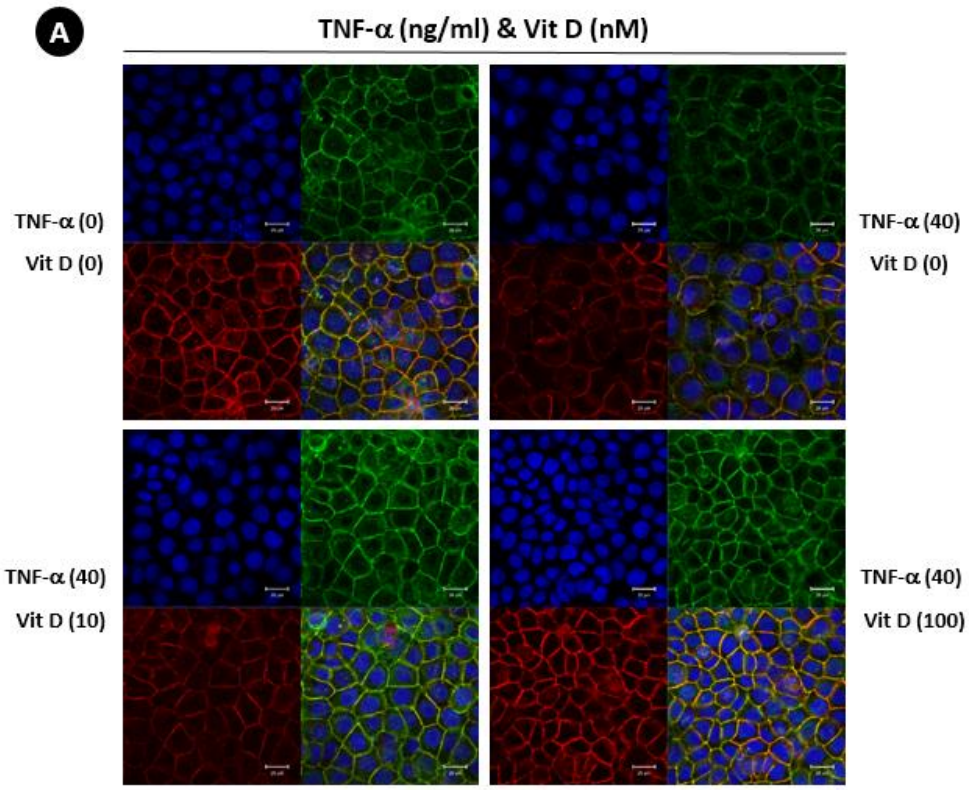

A
TNF-α (ng/ml)
0
10
40
100
B
Development of ECJ
1.0
0.8
0.6
0.4
0.2
0.0
a)
b)
b)
b)
-
10
40
100
TNF-α (ng/mL)

Figure 4. TNF-α suppresses the development of ECJs in HGKs *in vitro*. Cells were cultured for 24 hours for the establishment of the ECJs and then treated with various amounts of TNF-α (10, 40, 100 ng/mL). (A) ECJ development was detected by immunocytochemical staining of E-cadherin expression (red). In addition, F-actin (green) was stained with FITC-phalloidin and nuclei were stained with DAPI (blue). The lower right pictures in each set of pictures are the merged images of the E-cadherin, F-actin, and nuclei images. (B) The average ratio of the development of ECJs per cell was calculated.

TNF-α: tumor necrosis factor-alpha, ECJ: E-cadherin junction, HGK: human gingival keratinocyte, DAPI: 4′,6-diamidino-2-phenylindole dihydrochloride.

[a)] $P<0.05$ versus [b)](Nonparametric Kruskal-Wallis test and *post hoc* multiple comparison with the Dunn test)


A
TNF-α (ng/ml) & Vit D (nM)
TNF-α (0)
Vit D (0)
TNF-α (40)
Vit D (0)
TNF-α (40)
Vit D (10)
TNF-α (40)
Vit D (100)

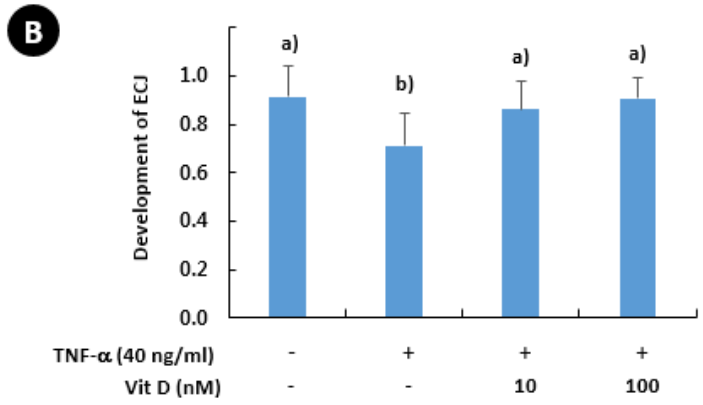
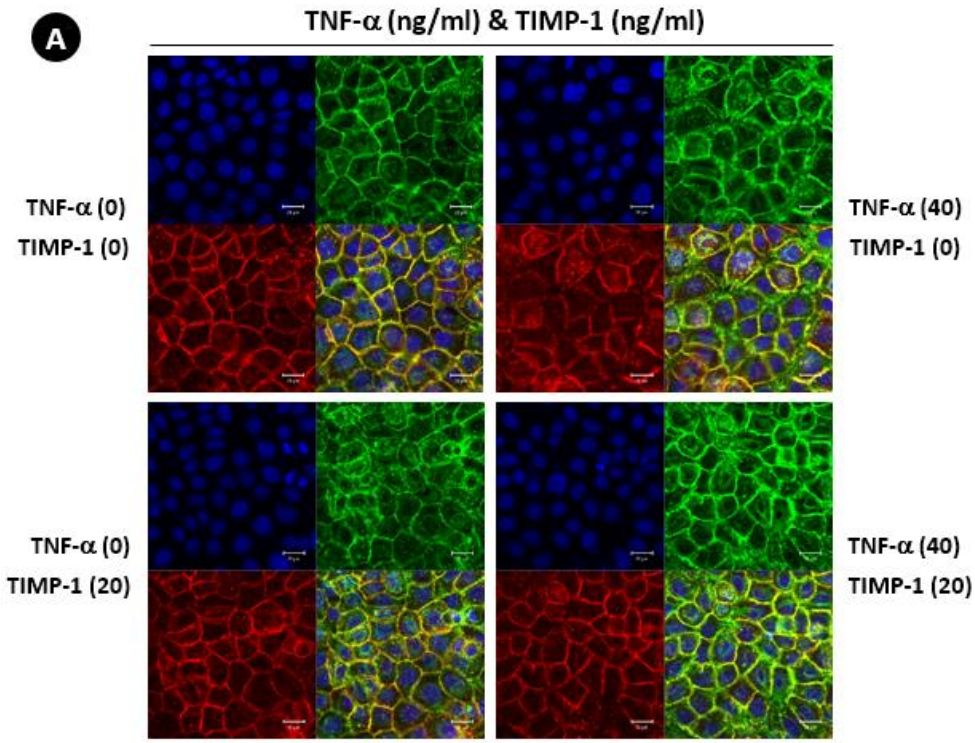

B
Development of ECJ
1.0
0.8
0.6
0.4
0.2
0.0
a)
b)
a)
a)
TNF-α (40 ng/ml) - + + +
Vit D (nM) - - 10 100

Figure 5. Vitamin D suppresses the dissociation of the ECJs induced by TNF-α in HGKs *in vitro*. Cells were cultured for 24 hours for the establishment of ECJs and pretreated with vitamin D (10, 100 nM) or without it for an additional 24 hours. Then, cells were cultured with or without TNF-α for 24 hours along with vitamin D. (A) ECJ development was detected by immunocytochemical staining of E-cadherin expression (red). In addition, F-actin (green) was stained with FITC-phalloidin and nuclei were stained with DAPI (blue). The lower right pictures in each set of pictures are the merged images of the E-cadherin, F-actin, and nuclei images. (B) The average ratio of the development of ECJs per cell was calculated.

ECJ: E-cadherin junction, TNF-α: tumor necrosis factor-alpha, HGK: human gingival keratinocyte, DAPI: 4′,6-diamidino-2-phenylindole dihydrochloride.

[a)]$P<0.05$ versus [b)](Nonparametric Kruskal-Wallis test and *post hoc* multiple comparison with the Dunn test)


A
TNF-α (ng/ml) & TIMP-1 (ng/ml)
TNF-α (0)
TIMP-1 (0)
TNF-α (40)
TIMP-1 (0)
TNF-α (0)
TIMP-1 (20)
TNF-α (40)
TIMP-1 (20)

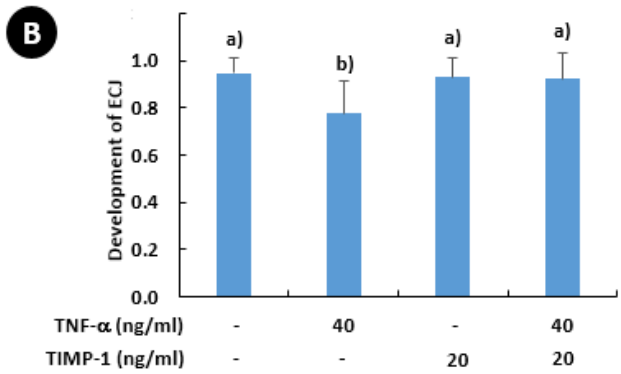
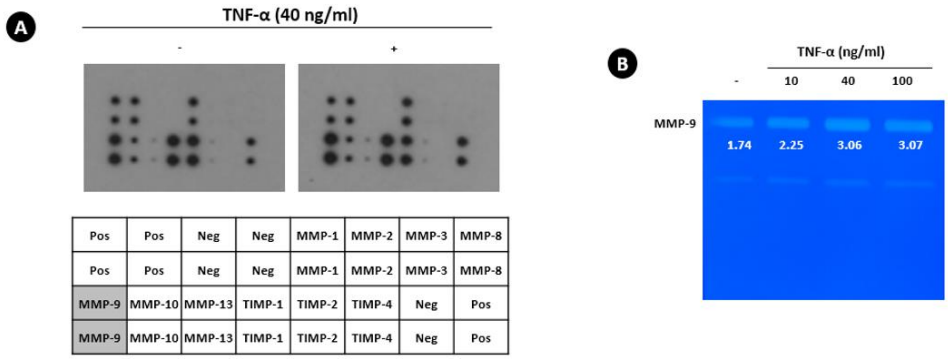

B
Development of ECJ
1.0
0.8
0.6
0.4
0.2
0.0
a)
b)
a)
a)
TNF-α (ng/ml) - 40 - 40
TIMP-1 (ng/ml) - - 20 20

Figure 6. TIMP-1 suppresses the dissociation of ECJs induced by TNF-α in HGKs *in vitro*. Cells were cultured for 24 hours for the establishment of the ECJs and then treated with TNF-α (40 ng/mL), with or without TIMP-1 (20 ng/mL) for an additional 24 hours. (A) ECJ development was followed by immunocytochemical staining of E-cadherin expression (red). In addition, F-actin (green) was stained with FITC-phalloidin and nuclei were stained with DAPI (blue). The lower right pictures in each set of pictures are the merged images of the E-cadherin, F-actin, and nuclei images. (B) The average ratio of the development of ECJs per cell was calculated by taking the mean of the values that were obtained by dividing the length of the E-cadherin expressed at cell junctions by the total perimeter of each cell.

TIMP-1: tissue inhibitor of metalloproteinase-1, ECJ: E-cadherin junction, TNF-α: tumor necrosis factor-alpha, HGK: human gingival keratinocyte, DAPI: 4′,6-diamidino-2-phenylindole dihydrochloride.

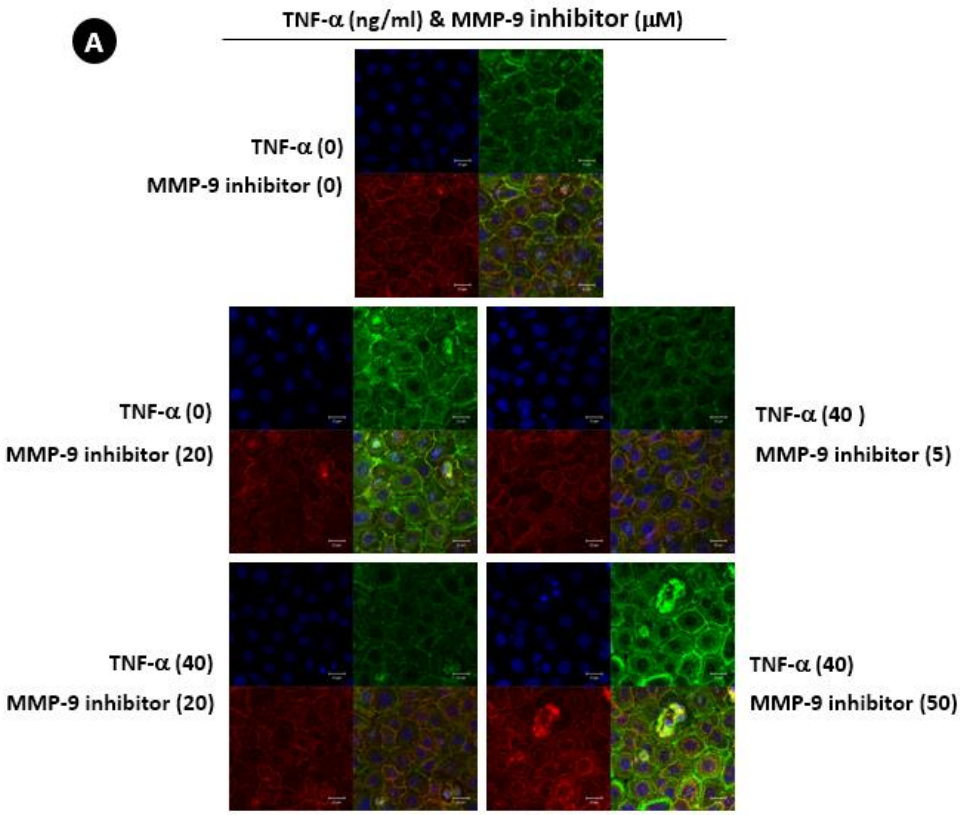
[a)] $P<0.05$ versus [b)](Nonparametric Kruskal-Wallis test and *post hoc* multiple comparison with the Dunn test)



Pos	Pos	Neg	Neg	MMP-1	MMP-2	MMP-3	MMP-8
Pos	Pos	Neg	Neg	MMP-1	MMP-2	MMP-3	MMP-8
MMP-9	MMP-10	MMP-13	TIMP-1	TIMP-2	TIMP-4	Neg	Pos
MMP-9	MMP-10	MMP-13	TIMP-1	TIMP-2	TIMP-4	Neg	Pos

Figure 7. TNF-α upregulates the secretion of MMP-9, as shown by antibody microarray analysis, and gelatinolytic digestion by the secreted MMP-9, as demonstrated by gelatin zymography in HGKs *in vitro*, respectively. (A) Cells were cultured for 24 hours for the establishment of the ECJs and then treated with TNF-α (40 ng/mL). Antibody microarray analysis of MMPs was performed by applying conditioned culture medium to the antibody microarray chip for MMPs. The table contains a list of the MMPs or TIMPs in the array. The numbers under the MMP-9 blots indicate the intensity of MMP-9 standardized using the positive controls as references (boxes). (B) Cells were cultured for 24 hours for the establishment of the ECJs and then treated with various amounts of TNF-α (10, 40, 100 ng/mL). Secretion of MMP-9 into the culture medium was assessed by gelatin zymogram analysis. The numbers under the MMP-9 bands indicate the activity of MMP-9.

TNF-α: tumor necrosis factor-alpha, MMP-9: matrix metalloproteinase-9, HGK: human gingival keratinocyte, ECJ: E-cadherin junction, Pos: positive control for the chip reactions, Neg: negative control for the chip reactions.


A
TNF-α (ng/ml) & MMP-9 inhibitor (μM)
TNF-α (0)
MMP-9 inhibitor (0)
TNF-α (0)
MMP-9 inhibitor (20)
TNF-α (40)
MMP-9 inhibitor (5)
TNF-α (40)
MMP-9 inhibitor (20)
TNF-α (40)
MMP-9 inhibitor (50)

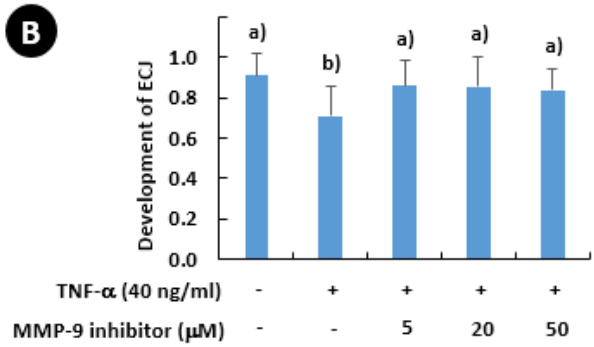
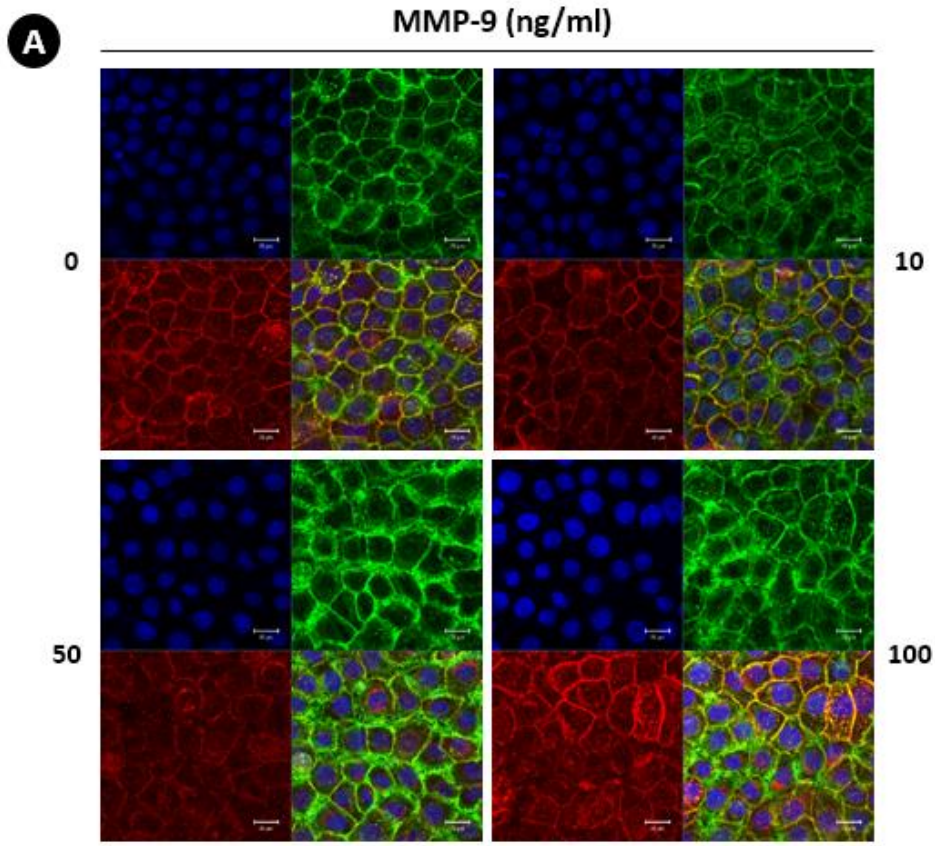

B
Development of ECJ
1.0
0.8
0.6
0.4
0.2
0.0
a)
b)
a)
a)
a)
TNF-α (40 ng/ml) - + + + +
MMP-9 inhibitor (μM) - - 5 20 50

Figure 8. A specific inhibitor of MMP-9 reverses the effect of TNF-α on the development of ECJs in HGKs *in vitro*. Cells were cultured for 24 hours for the establishment of the ECJs and then treated with TNF-α (40 ng/mL) along with various amounts of an MMP-9 inhibitor (5, 20, or 50 μM) or without it for an additional 24 hours. (A) ECJ development was followed by immunocytochemical staining of E-cadherin expression (red). In addition, F-actin (green) was stained with FITC-phalloidin and nuclei were stained with DAPI (blue). The lower right pictures in each set of pictures are the merged images of the E-cadherin, F-actin, and nuclei images. (B) The average extent of the development of ECJs per cell was calculated by taking the mean of the values that were obtained by dividing the length of the E-cadherin expressed at cell junctions by the total perimeter of each cell.

MMP-9: matrix metalloproteinase-9, TNF-α: tumor necrosis factor-alpha, ECJ: E-cadherin junction, HGK: human gingival keratinocyte, DAPI: 4′,6-diamidino-2-phenylindole dihydrochloride.

[a)]$P<0.05$ versus [b)](Nonparametric Kruskal-Wallis test and *post hoc* multiple comparison with the Dunn test)


A
MMP-9 (ng/ml)
0
10
50
100

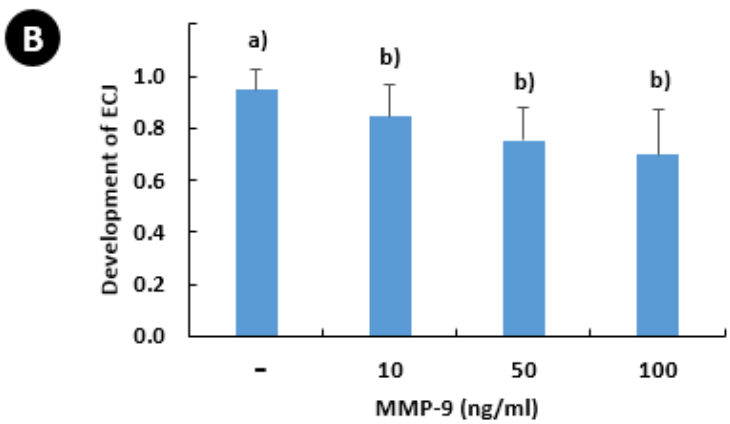

B
a)
b)
b)
b)
1.0
0.8
0.6
0.4
0.2
0.0
Development of ECJ
-
10
50
100
MMP-9 (ng/ml)

Figure 9. MMP-9 induces the dissociation of ECJs in HGKs *in vitro*. Cells were cultured for 24 hours for the establishment of the ECJs and then treated with various amounts of MMP-9 (10, 50, 100 ng/mL). (A) ECJ development was followed by immunocytochemical staining of E-cadherin expression (red). In addition, F-actin (green) was stained with FITC-phalloidin and nuclei were stained with DAPI (blue). The lower right pictures in each set of pictures are the merged images of the E-cadherin, F-actin, and nuclei images. (B) The average extent of the development of ECJs per cell was calculated by taking the mean of the values that were obtained by dividing the length of the E-cadherin expressed at cell junctions by the total perimeter of each cell.

MMP-9: matrix metalloproteinase-9, ECJ: E-cadherin junction, HGK: human gingival keratinocyte, DAPI: 4′,6-diamidino-2-phenylindole dihydrochloride.

[a)] $P<0.05$ versus [b)](Nonparametric Kruskal-Wallis test and *post hoc* multiple comparison with the Dunn test)

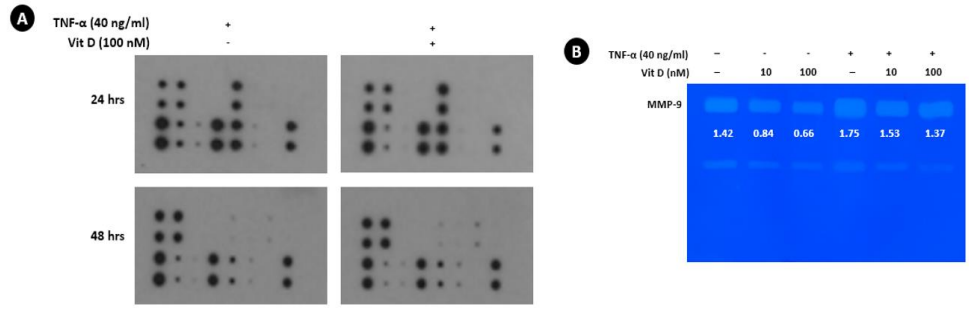
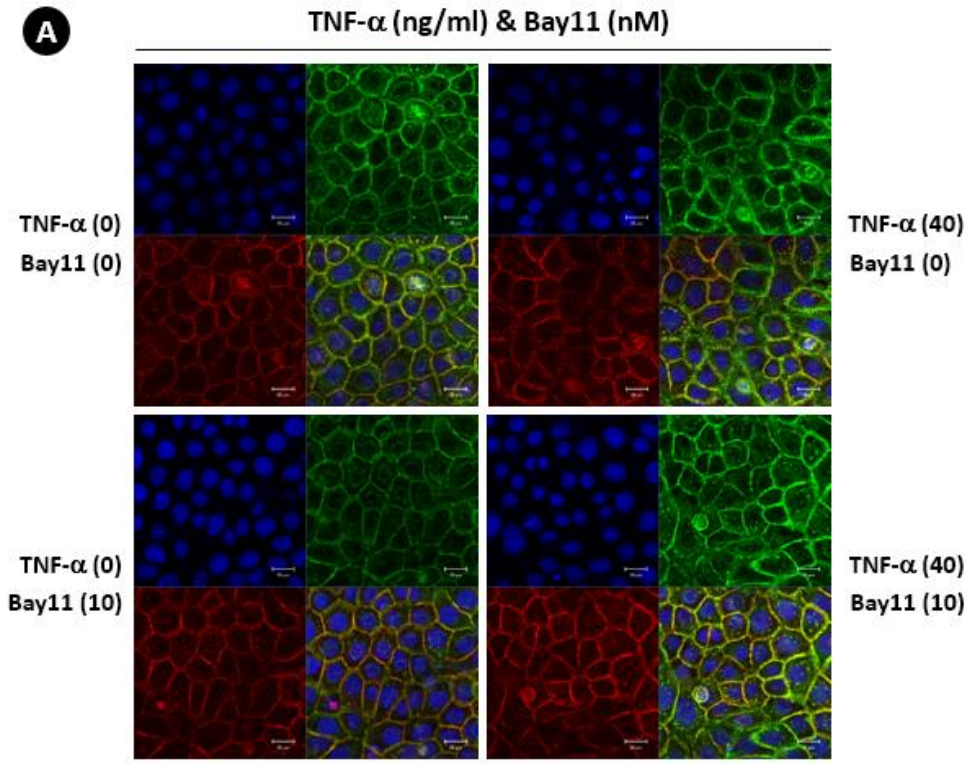


Figure 10. Vitamin D downregulates the TNF-α-induced secretion of MMP-9 and the gelatinolytic digestion by the MMP-9 upregulated by TNF-α in HGKs *in vitro*. Cells were cultured for 24 hours for the establishment of ECJs and pretreated with or without vitamin D (10 or 100 nM) for an additional 24 hours. Then, cells were cultured with or without TNF-α (40 ng/mL) along with vitamin D for 24 and 48 hours (A) or 48 hours (B). (A) Antibody microarray analysis of MMPs was performed by applying conditioned culture medium to the antibody microarray of MMPs. Details on the arrays are shown in the legend of Figure 4A. The numbers under the MMP-9 blots indicate the intensity of MMP-9 standardized using the positive controls as references (boxes). (B) Secretion of MMP-9 into the culture medium was assessed by gelatin zymogram analysis. The numbers under the MMP-9 bands indicate the activity of MMP-9.

TNF-α: tumor necrosis factor-alpha, MMP-9: matrix metalloproteinase-9, HGK: human gingival keratinocyte, ECJ: E-cadherin junction.


A
TNF-α (ng/ml) & Bay11 (nM)
TNF-α (0)
Bay11 (0)
TNF-α (40)
Bay11 (0)
TNF-α (0)
Bay11 (10)
TNF-α (40)
Bay11 (10)

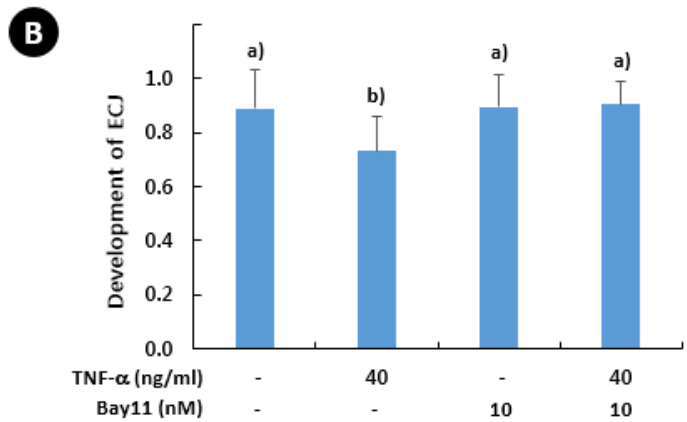

B
Development of ECJ
1.0
0.8
0.6
0.4
0.2
0.0
a)
b)
a)
a)
TNF-α (ng/ml) - 40 - 40
Bay11 (nM) - - 10 10

Figure 11. NF-κB inhibition by Bay 11-7082 reverses the TNF-α-induced effects on HGKs *in vitro*. Cells were cultured for 24 hours for the establishment of ECJs and then treated with TNF-α (40 ng/mL) along with Bay 11-7082 (Bay11; 10 nM) or without it for an additional 24 hours. (A) ECJ development was followed by immunocytochemical staining of E-cadherin expression (red). In addition, F-actin (green) was stained with FITC-phalloidin and nuclei were stained with DAPI (blue). The lower right pictures in each set of pictures are the merged images of the E-cadherin, F-actin, and nuclei images. (B) The average ratio of the development of ECJs per cell was calculated by taking the mean of the values which were obtained by dividing the length of the E-cadherin expressed at cell junctions by the total perimeter of each cell.

NF-κB: nuclear factor kappa B, TNF-α: tumor necrosis factor-alpha, HGK: human gingival keratinocyte, ECJ: E-cadherin junction, DAPI: 4′,6-diamidino-2-phenylindole dihydrochloride.

[a)] $P<0.05$ versus [b)](Nonparametric Kruskal-Wallis test and *post hoc* multiple comparison with the Dunn test)

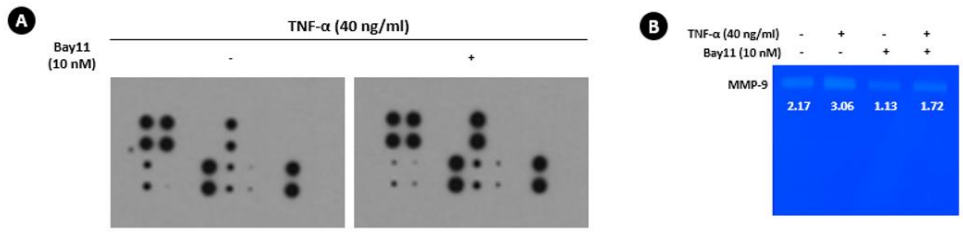
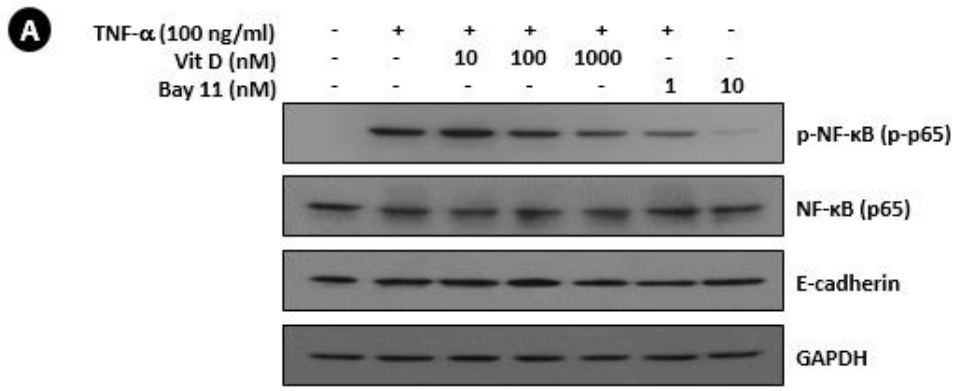


Figure 12. The inhibition of NF-κB signaling by Bay 11-7082 lowers the secretion of MMP-9 into the culture medium and the gelatinolytic digestion by the secreted MMP-9, upregulated by TNF-α. Cells were cultured for 24 hours for the establishment of ECJs and then treated with TNF-α (40 ng/mL) along with Bay 11-7082 (Bay11; 10 nM) or without it for an additional 24 hours. (A) Antibody microarray analyses of MMPs were performed by applying culture medium to the MMPs antibody microarray chip. Representative data from the triplicate measurements are shown. Details on the arrays are shown in the legend of Figure 4A. The numbers under the MMP-9 blots indicate the intensity of MMP-9 standardized using the positive controls as references (boxes). (B) Secretion of MMP-9 into the culture medium was assessed by gelatin zymogram analysis. The numbers under the MMP-9 bands indicate the activity of MMP-9.

NF-κB: nuclear factor kappa B, MMP-9: matrix metalloproteinase-9, TNF-α: tumor necrosis factor-alpha, ECJ: E-cadherin junction.


A
TNF-α (100 ng/ml) - + + + + + -
Vit D (nM) - - 10 100 1000 - -
Bay 11 (nM) - - - - - 1 10
p-NF-κB (p-p65)
NF-κB (p65)
E-cadherin
GAPDH

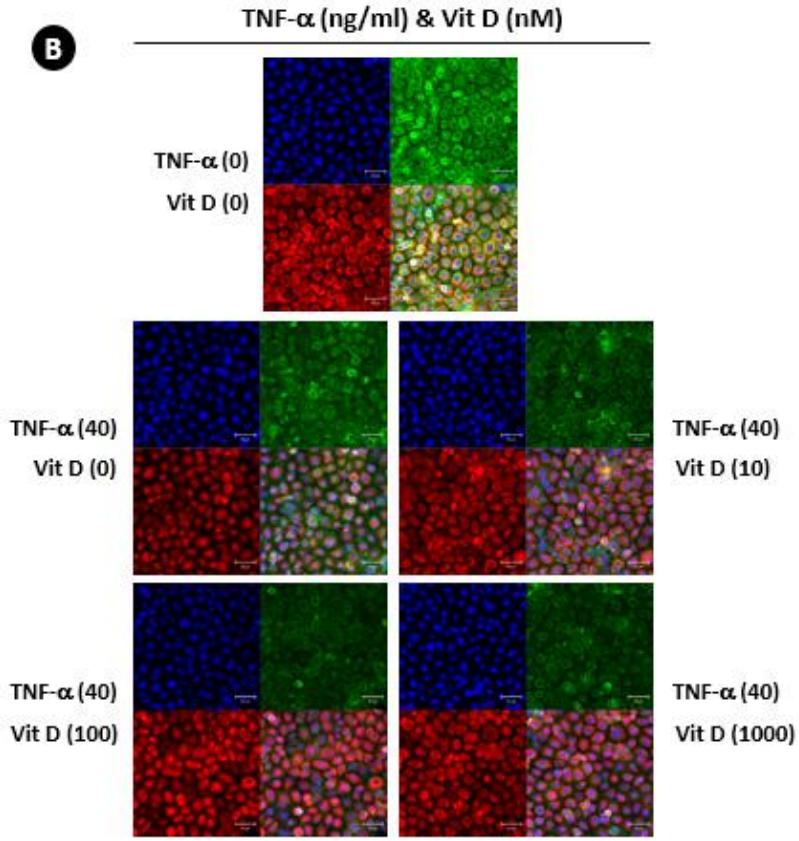

B
TNF-α (ng/ml) & Vit D (nM)
TNF-α (0)
Vit D (0)
TNF-α (40)
Vit D (0)
TNF-α (40)
Vit D (10)
TNF-α (40)
Vit D (100)
TNF-α (40)
Vit D (1000)

Figure 13. Vitamin D inhibits TNF-α-induced NF-κB signaling, as shown by western blotting. Cells were cultured for 48 hours and then pretreated with vitamin D (10, 100, or 1,000 nM) or without it for 24 hours. Before cell lysis for western blotting, cells were treated with TNF-α (100 ng/mL) for 10 minutes. Bay 11-7082 (Bay 11; 1 nM), a pharmacological inactivator of NF-κB signaling, was used as an internal control of the immunoblotting analyses to show that TNF-α-induced NF-κB signaling was reduced when NF-κB signaling was pharmacologically inactivated in HGKs. (A) Vitamin D inhibits TNF-α induced NF-κB translocation to the nucleus in vitro. Cells were cultured for 24 hours and pretreated with vitamin D (10, 100, and 1000 nM) or maintained without vitamin D for additional 24 hours. NF-κB expression was detected by immunocytochemical staining (red). F-actin (green) was stained with FITC-phalloidin and nuclei were stained with DAPI (blue). TNF-α increased NF-κB translocation to the nucleus, whereas pretreatment with vitamin D reduced NF-κB translocation dramatically. (B) TNF-α: tumor necrosis factor-alpha, NF-κB: nuclear factor kappa B, HGK: human gingival keratinocyte, GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

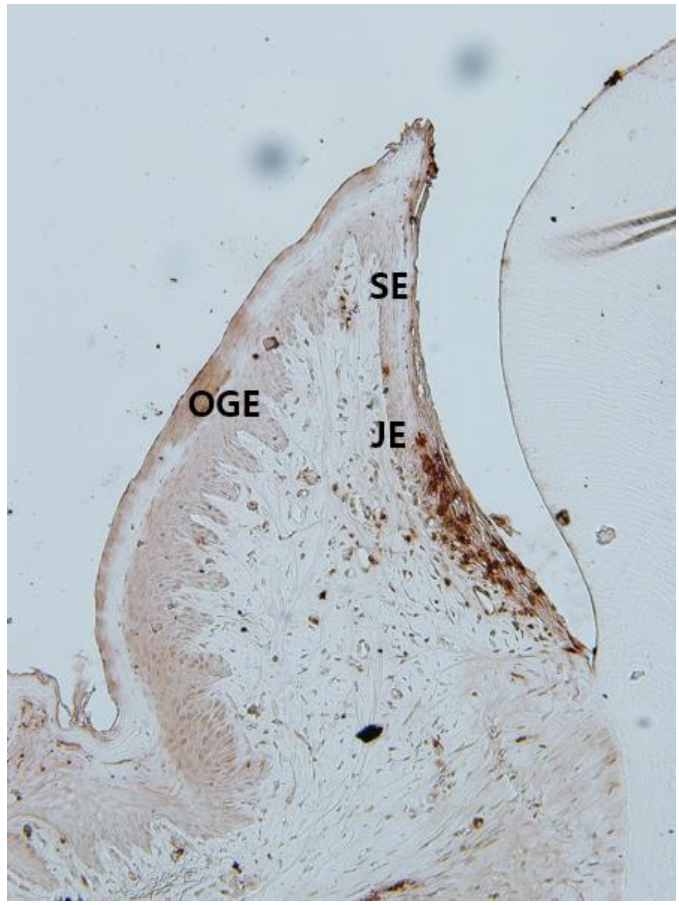


Figure 14. VDR is expressed in the gingival epithelium. VDR expression in gingival epithelium surrounding rat mandibular molars was examined using immuno-histochemical methods. Expression of VDR was apparent in the JE (arrows). Expression of VDR was also localized in the OGE and SE.

VDR: vitamin D receptor, JE: junctional epithelium, OGE: oral gingival epithelium, SE: sulcular epithelium.

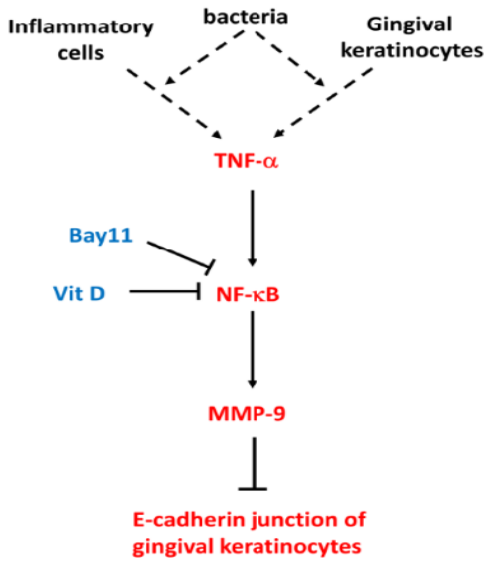


Figure 15. Diagram of a hypothetical molecular mechanism by which vitamin D suppresses the dissociation of ECJs induced by TNF-α. Vitamin D suppresses the NF-κB activation induced by TNF-α, which abolishes the detrimental action of TNF-α through downregulating the production of MMP-9, which breaks the integrity of ECJs. TNF-α may be produced by gingival keratinocytes infected with bacteria in an autocrine fashion at the early epithelial stage of infection [7] or could be externally provided from inflammatory cells in connective tissue at the late stage of inflammation. In summary, the present study suggests that vitamin D may be protective for periodontal health by strengthening the epithelial barrier.

ECJ: E-cadherin junction, TNF-α: tumor necrosis factor-alpha, NF-κB: nuclear factor kappa B, MMP-9: matrix metalloproteinase-9.

국문초록

치은 각화상피세포에서의 E-cadherin 결합을 보호하는 비타민 D의 역할

서울대학교 대학원 세포및발생생물학 전공

지도교수: 김현만

오창석

치주 건강에서 비타민 D의 항 염증 효과는 잘 알려져 있지만, 그 작용 기전은 완전히 밝혀져 있지 않다. 본 연구에서는 치은각화상피세포에서 E-cadherin 접합 강화에 대한 비타민 D의 효과에 대해 연구하였다. 구강 조직에서 상피 장벽은 미생물이나 독소의 침입을 막는데 필수적이기 때문에, 세포 간 온전한 접합을 유지하는 것이 중요하다. 치은 접합 상피는 특징적으로 세포 간 접합이 산발적이고 느슨하여 쉽게 세균이 침투할 수 있다. 이 접합 상피에서의 주요한 세포 간 접합의 유형은 E-cadherin 접합이다. 본 연구는 불멸화된 정상 사람

치은 각화상피세포주인 HOK-16B 세포를 사용하였고, 염증 환경 모방을 위해 세포를 TNF-α로 처리하였다. 배양액에 함유된 MMP-9의 정량적 확인을 위해서 MMP antibody microarray 방법과 zymography 방법을 사용하였다. 세포 내 분자의 발현 분석은 immunoblotting 방법을 사용하였다. 세포 간 접합의 발달 정도는 면역세포화학법으로 E-cadherin을 염색하여 각 세포에 발달한 E-cadherin 접합의 상대적인 발달 정도를 측정하였다. E-cadherin 접합의 상대적인 발달 정도는 각 세포에서 E-cadherin 접합의 길이를 각 세포의 전체 접합의 길이로 나누어 구하였다. 치은에서 비타민 D 수용체의 발현은 면역조직화학법으로 조사하였다. 연구 결과로 TNF-α는 치은각화상피세포에서 E-cadherin 접합의 발달을 저해하였다. TNF-α에 의한 E-cadherin 접합의 발달 저해는 MMP-9 생산 증가에 의해 일어나고, 이 현상은 MMP-9의 억제제를 처리함에 의해서 억제됨을 확인하였다. 더불어, E-cadherin 접합의 발달은 외인성의 MMP-9에 의해서 저하되었다. 한편 비타민 D는 TNF-α에 의한 MMP-9의 생산 증가를 감소시킴으로써, E-cadherin 접합의 발달 저해를 감소시켰다. 치은각화상피세포에서 TNF-α에 의한 E-cadherin 접합의 발달 저하는 nuclear factor kappa B (NF-κB) 신호전달을 억제함으로써 피할 수 있었는데, 비타민 D도 역시 TNF-α에 의한 NF-κB 신호전달 활성화를 억제하였다. 비타민 D 수용체는 접합상피를 포함하여 치은상피조직에서

뚜렷하게 발현되었다. 이러한 결과는 TNF-α가 사람치은각화상피세포에서 MMP-9 생산을 증가시켜 E-cadherin 접합의 발달을 저해하는데, 비타민 D는 TNF-α에 의한 MMP-9의 생산 증가를 억제함으로써 E-cadherin 접합의 발달 저하를 막을 수 있음을 보여준다. 또한 이러한 비타민 D의 효과는 TNF-α에 의한 NF-κB 신호전달의 활성화를 억제함으로써 가능함을 보여준다. 이로서 본 연구는 비타민 D가 상피 방어벽을 강화시켜 박테리아 침입으로부터 치주조직을 보호할 수 있음을 시사한다.

주요어: E-cadherin, 각화상피세포, Matrix metalloproteinase 9, NF-kappa B, Tumor necrosis factor-alpha, 비타민 D

학 번: 2015-31243