

## CBT-SL5, a Bacteriocin from *Enterococcus faecalis*, Suppresses the Expression of Interleukin-8 Induced by *Propionibacterium acnes* in Cultured Human Keratinocytes

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*Propionibacterium acnes* is known to play a pivotal role in the pathogenesis of acne vulgaris. CBT-SL5 is one of the antimicrobial peptides from *Enterococcus faecalis* SL5, and it has shown antimicrobial activity against *P. acnes*. The aim of this study was to investigate the anti-inflammatory effect of CBT-SL5 on the inflammation induced by *P. acnes* in cultured human keratinocytes. Cultured human keratinocytes derived from neonatal foreskin were treated with heat-killed *P. acnes* to induce inflammation, and then various concentrations of CBT-SL5 were added to the *P. acnes*-treated keratinocytes. The mRNA expression and protein secretion of interleukin (IL)-8, an inflammation marker, was analyzed by real-time reverse transcription polymerase chain reaction and enzyme-linked immunosorbent assay, respectively. We also analyzed the nuclear factor-kappa B (NF- $\kappa$ B) p65 translocation by performing immunofluorescent staining. *P. acnes* treatment upregulated the IL-8 mRNA expression in the keratinocytes, and this was brought about through both toll-like receptor (TLR)2 and TLR4. At the concentrations of 10, 50, and 100 ng/ml, CBT-SL5 significantly downregulated the *P. acnes*-induced IL-8 mRNA expression and protein production ( $p < 0.05$ ). At 6 h and 12 h of the treatment, CBT-SL5 significantly suppressed the *P. acnes*-induced IL-8 mRNA expression. Secretion of IL-8 protein was significantly reduced at 24 h. The functional inhibitory activity of CBT-SL5 was shown by CBT-SL5 suppressing the *P. acnes*-induced NF- $\kappa$ B translocation from the cytoplasm to the nucleus. These results demonstrated that CBT-SL5 suppressed the *P. acnes*-induced IL-8 expression in keratinocytes. Therefore, CBT-SL5 may be a novel anti-inflammatory treatment for acne.

**Keywords:** Anti-inflammatory, bacteriocin, CBT-SL5, *Enterococcus faecalis*, IL-8, *Propionibacterium acnes*

*Propionibacterium acnes* is a Gram-positive, anaerobic bacterium that is part of the normal skin flora [5]. This bacterium is thought to play a central role in the inflammation of acne vulgaris [12]. Recent reports have suggested that *P. acnes* may contribute to inflammation in acne through activation of the toll-like receptors (TLRs), especially TLR2 and TLR4, which are expressed on nonspecific immune cells (keratinocytes, sebocytes, and monocytes), and this in turn would lead to release of proinflammatory cytokines such as interleukin(IL)-1 $\alpha$ , IL-6, IL-8, and tumor necrosis factor (TNF)- $\alpha$  [13, 15]. *P. acnes*-induced IL-8 secretion could have a key role in the initiation of the inflammatory events in acne [20]. IL-8 is a major inflammatory mediator and a strong chemotactic factor for neutrophils, basophils, and lymphocytes [33]. Previous studies have provided evidence that cultured keratinocytes produce IL-8 in a time-dependent manner in response to *P. acnes* treatment and this process may be mediated by the activation of the transcription factor nuclear factor-kappa B (NF- $\kappa$ B) [3, 31]. The currently available topical agents for the treatment of acne include clindamycin, erythromycin, and benzoyl peroxide. However, these topical therapeutic agents have been known to induce some side effects, such as skin irritation or appearance of resistant bacteria [5–7]. Therefore, many researchers have tried to develop new therapeutic agents for acne that have a higher therapeutic activity, but few side effects [21, 29]. Antimicrobial peptides (AMPs) have a broad spectrum of antimicrobial activity, kill target cells rapidly, and are active against clinically relevant pathogens. Lactic acid bacteria produce a variety of AMPs called bacteriocins [9, 16]. These bacteriocins are now worthy of note as therapeutic agents because they are made by nonpathogenic bacteria that normally colonize the human body and they can be easily digested by the protease from gastric organs [11].

CBT-SL5 is one of the bacteriocins derived from *Enterococcus faecalis* SL5. The molecular mass of CBT-

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SL5 is 4.5 kDa and is made up of 44–46 amino acid residues. The N-terminal peptide sequence of CBT-SL5 was given as follows: Ala-Ile-Ala-Leu-Val-Ala. In our previous *in vitro* study, the spectrum of antimicrobial activity of CBT-SL5 against 4 kinds of bacterial strains (*P. acnes*, *Bacillus cereus*, *Staphylococcus aureus*, and *Bacillus subtilis*) was tested by the spot-on-the-lawn method as described by Van Reenen *et al.* [30]. The results showed that CBT-SL5 had an inhibitory activity against all of the tested bacterial strains, and the *P. acnes* strains were much more sensitive to CBT-SL5 than were the other strains (B.S. Kang *et al.* 2007. Abstr. Kor. Soc. Acne Res. Sym, p. 43–44).

Recent studies have shown that in addition to their antimicrobial action, AMPs can inhibit the inflammation triggered by bacteria. The anti-inflammatory activity of AMPs in acne vulgaris can be explained by their ability to bind proinflammatory bacterial factors [e.g., lipoteichoic acid (LTA) and lipopolysaccharide (LPS)], which sequesters them from the site of inflammation, and so this inhibits the secretion of proinflammatory cytokines (e.g., TNF- $\alpha$ , IL-1) by the host cells [17].

The aim of this study was to examine the anti-inflammatory effect of CBT-SL5 on the inflammation induced by *P. acnes* in cultured human keratinocytes.

## MATERIALS AND METHODS

### *Propionibacterium acnes* Culture

*P. acnes* KCTC 3314 was obtained from the Korean Collection for Type Culture and cultured on BL broth media at 37°C under anaerobic conditions for 24 h. The cells were collected by centrifugation at 1,800  $\times$ g for 5 min at 20°C; they were then washed twice with PBS (pH 7.4). After the final washing, the cell pellets were resuspended in PBS and the cells were killed by heating at 80°C for 30 min. The heat-killed *P. acnes* was aliquoted and stored at 4°C until use.

### Preparation of CBT-SL5

CBT-SL5 is a bacteriocin from *E. faecalis* SL-5, which was a generous gift by Cell Biotech Co., Ltd. (Incheon, Korea). The stock solution was made by mixing 1 g of CBT-SL5 per 1 ml of keratinocyte growth media (KGM) (Cambrex, Walkersville, MD, U.S.A.) and this was filtered through a 0.20- $\mu$ m-pore-size filter (Toyo Rochi Kaisha, Ltd., Tokyo, Japan). The filtered solution was stored at 4°C until use.

### Keratinocyte Culture

Human epidermal keratinocytes from neonatal foreskin were purchased from Modern Cell and Tissue Technologies (Seoul, Korea) and these cells were cultured in KGM supplemented with 7.5 mg/ml bovine pituitary extract, 0.1 ng/ml human recombinant epidermal growth factor, 5  $\mu$ g/ml insulin, 0.5  $\mu$ g/ml hydrocortisone, 50  $\mu$ g/ml gentamicin, and 50 ng/ml amphotericin-B for 2 or 3 days at 37°C in 5% CO<sub>2</sub> and 95% humidity. The keratinocytes were

seeded in a 100-mm culture dish coated by type collagen. The medium was changed three times per week until the cells reached 80% confluency. After that time, the cells were subcultured.

### Treatment of *P. acnes* and/or CBT-SL5

The keratinocytes were used at passage 2, 3, or 4 and they were seeded onto 12-well cell culture plates (Nunc, Naperville, IL, U.S.A.) at a density of 3  $\times$  10<sup>4</sup> cells per well. When the cells reached 70% confluency, the medium was changed to medium that did not have hydrocortisone, gentamicin, and amphotericin-B, and the cells were next incubated overnight. The next day, the keratinocytes were stimulated with heat-killed *P. acnes* (5  $\times$  10<sup>7</sup> or 1  $\times$  10<sup>8</sup> bacteria/ml) and then incubated for 6 h at 37°C in an atmosphere of 5% CO<sub>2</sub>. Various concentrations of CBT-SL5 (10, 50, or 100 ng/ml) were added to each well, and the cells were incubated for 6 and 24 h. In some experiments, 100 ng/ml CBT-SL5 was added to the keratinocytes treated with an optimal concentration of *P. acnes* and these cells were incubated for 3, 6, 12, and 24 h. For a negative control, keratinocytes were cultured with KGM without any stimulants. Following incubation, the cells were harvested and the supernatants at each time were collected by centrifugation at 14,000  $\times$ g, 4°C, for 10 min to obtain the cell-free sample. The supernatants were aliquoted and then stored at -20°C before IL-8 assay.

### Immunocytochemistry for TLR2 and TLR4

The keratinocytes were plated on a Lab-Tek 4-well chamber slide (Nunc), and cultured for 6 h with heat-killed *P. acnes*. As a positive control, 10  $\mu$ g/ml of LTA from *S. aureus* (Sigma, St. Louis, MO, U.S.A.) or 10  $\mu$ g/ml of LPS from *Escherichia coli* (Sigma) was used. After removal of the culture medium, the cells were washed with PBS and fixed with 1% paraformaldehyde in 0.1 M PB for 30 min. After washing in PBS, the slides were placed in blocking buffer (1% BSA in PBS) at room temperature for 15 min. The samples were incubated with anti-TLR2 mouse monoclonal antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or anti-TLR4 mouse monoclonal antibody (1:100; Santa Cruz Biotechnology) diluted in PBS, respectively, overnight at 4°C. The next day, the samples were washed 3 times. The cells were then incubated with a biotinylated goat anti-mouse antibody (1:200; Molecular Probes, Eugene, OR, U.S.A.) at room temperature for 1 h. After washing cells with PBS, the slides were incubated with NovaRed (Vector Laboratories, Burlingame, CA, U.S.A.) for 5 min. The reaction was stopped by washing with distilled water for 10 min and then counterstaining was done with hematoxylin (Vector Laboratories) for 1 min. The slides were rinsed with distilled water and mounted in 40% glycerol and the edge of each cover glass was sealed with regular transparent nail polish; this was allowed to dry for 10 min. The slides were analyzed using a microscope.

### Blocking of TLR2 and TLR4

Cultured keratinocytes were grown on 12-well plates and pre-treated with anti-human TLR2 antibody (10  $\mu$ g/ml; eBioscience, San Diego, CA, U.S.A.) or anti-human TLR4 antibody (10  $\mu$ g/ml; eBioscience) for 1 h. Then, *P. acnes* (1  $\times$  10<sup>8</sup> bacteria/ml), LTA (10  $\mu$ g/ml), or LPS (10  $\mu$ g/ml) was added to the keratinocytes, respectively. After 3-h stimulation, the keratinocytes were harvested for real-time reverse transcription polymerase chain reaction (RT-PCR) analysis.

**Table 1.** Sequences of primers used for real-time RT-PCR.

Primer	Direction	Sequences
TLR2	Forward	5'-TGTGAACCTCCAGGCTCTG-3'
	Reverse	5'-CTCCATATTATCCCCTCTCAGG-3'
TLR4	Forward	5'-ACAGAAGCTGGTGGCTGTG-3'
	Reverse	5'-TCTTTAAATGCACCTGGTTGG-3'
IL-8	Forward	5'-CCACACTGCGCCAACA-3'
	Reverse	5'-GCATCTTCACTGATTCTTGGAT-3'
β-Actin	Forward	5'-TCTACTATGAGCTGCGTGTG-3'
	Reverse	5'-GGTGAGGATCTTCATGAGGT-3'

#### Quantification of the mRNA Expression Using Real-Time RT-PCR

The cells were detached from the 12-well culture plate using a cell scraper. The total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.), according to the manufacturer's instructions. Three µg of total RNA was reverse transcribed to cDNA for 2 h at 42°C using 200 U/µg Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI, U.S.A.), 1 µl of oligo(dT)<sub>15</sub> primer (Promega), and 2 µl of 10 mM dNTP mixture per 20 µl of final volume.

One µl of cDNA was analyzed by real-time RT-PCR in a MX3000P (Stratagene, La Jolla, CA, U.S.A.) using iQ SYBR Green supermix (Bio-Rad, Hercules, CA, U.S.A.). The sequences of the PCR primer pairs (5'-3') used in this study are given in Table 1. The amplification protocol contained one cycle of initial denaturation at 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 30 sec, annealing at 61°C for 30 sec, extension at 72°C for 30 sec, and one cycle of terminal extension at 95°C for 1 min. β-Actin was used as a housekeeping gene to normalize the quantitative RT-PCR cytokine levels. All the experiments were performed in duplicate.

#### Enzyme-Linked Immunosorbent Assay (ELISA) for IL-8 Protein

A BD OptEIA human IL-8 ELISA kit II (BD Biosciences, San Diego, CA, U.S.A.) was used to determine the IL-8 levels in the collected supernatants according to the manufacturer's instructions. Briefly, 50 µl of sample diluent and 100 µl of the standards/sample was added to a 96-well plate coated by monoclonal IL-8 antibody and this was incubated for 2 h at room temperature. The contents of wells were decanted and repeatedly washed with wash buffer at least 5 times. It was important that complete removal of liquid was done. One hundred µl of working detector was added to each well and this was incubated for 1 h at room temperature. The liquids of the wells were aspirated and the wells were washed 7 times with wash buffer. Then, 100 µl of TMB one-step substrate reagent was added to each well, and this was incubated for 30 min at room temperature. Fifty µl of stop solution was added for halting the reaction. Reading of the absorbance at 450 nm was done by an ELISA reader (Spectra Max250; Molecular Devices Co., U.S.A.). All the experiments were performed in duplicate.

#### Immunofluorescence Analysis for NF-κB Nuclear Translocation

The keratinocytes were plated on a Lab-Tek 4-well chamber slide (Nunc), and cultured for 30 min with heat-killed *P. acnes* in the presence or absence of CBT-SL5. Ten µg/ml LTA was used as a positive control. PBS was used as a negative control. After removal of the culture medium, the cells were washed with PBS and fixed with

ice-cold acetone:methanol (1:1) at -20°C for 10 min. After washing in PBS, the slides were placed in blocking buffer (1% BSA in PBS) at room temperature for 30 min to minimize nonspecific adsorption of antibodies to the slide. The samples were incubated with NF-κB p65 rabbit polyclonal antibody (1:100; Santa Cruz Biotechnology) diluted in PBS at room temperature for 1 h. The cells were then incubated with an Alexa 488-conjugated goat anti-rabbit antibody (1:1,000; Molecular Probes, Eugene, OR, U.S.A.) at room temperature for 1 h. After washing the cells with PBS, the slide was mounted in 40% glycerol and the edge of each cover glass was sealed with regular transparent nail polish; this was allowed to dry for 10 min. The slides were analyzed using an inverted fluorescence microscope (Axiovert 200MAT; Carl Zeiss, Göttingen, Germany).

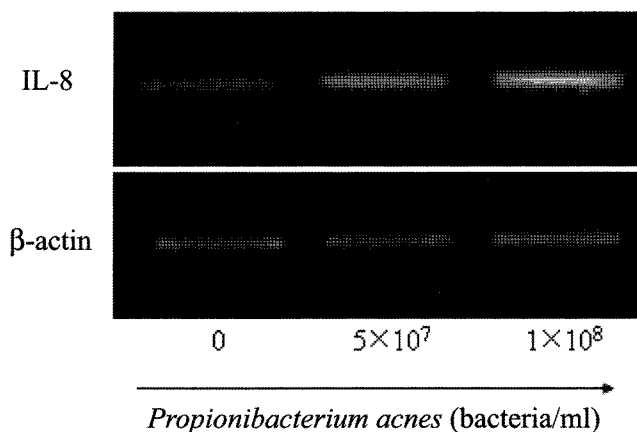
#### Statistical Analysis

The experimental results are expressed as means ± the standard errors. The data was compared using one-way analysis of variance (ANOVA) followed by Duncan post-hoc test to determine statistical differences after multiple comparisons (SAS; SAS Institute Inc., NC, U.S.A.). *P* values <0.05 were considered statistically significant.

## RESULTS

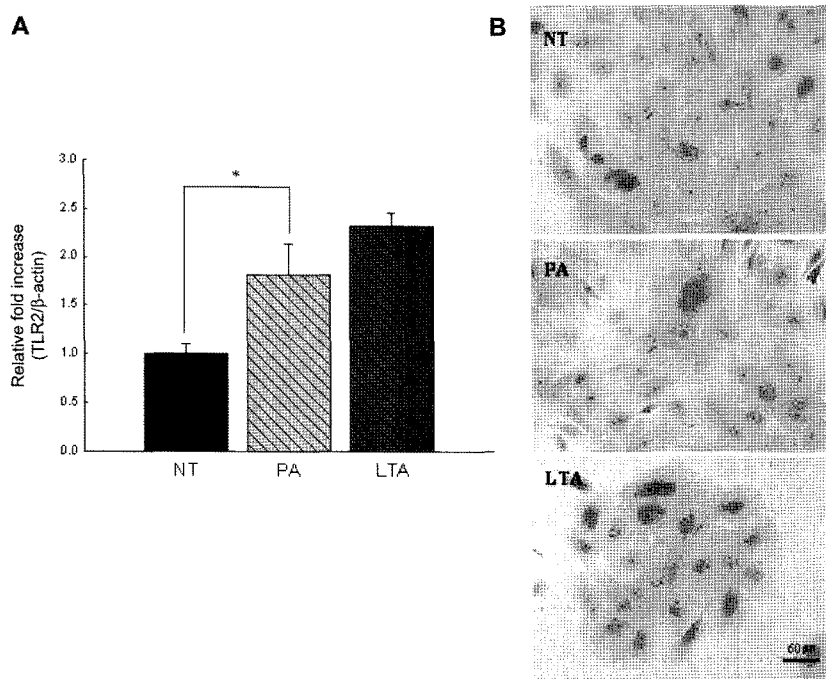
### *P. acnes* Treatment Increased the IL-8 mRNA Expression in the Keratinocytes

To obtain an optimal concentration of *P. acnes* that induces an IL-8 mRNA expression, the keratinocytes were incubated with heat-killed *P. acnes* at concentrations of  $5 \times 10^7$  and  $1 \times 10^8$  bacteria/ml for 6 h, respectively. The IL-8 mRNA expression was analyzed by RT-PCR. As shown in Fig. 1, IL-8 mRNA was constitutively expressed in the cultured keratinocytes. *P. acnes* treatment increased the IL-8 mRNA

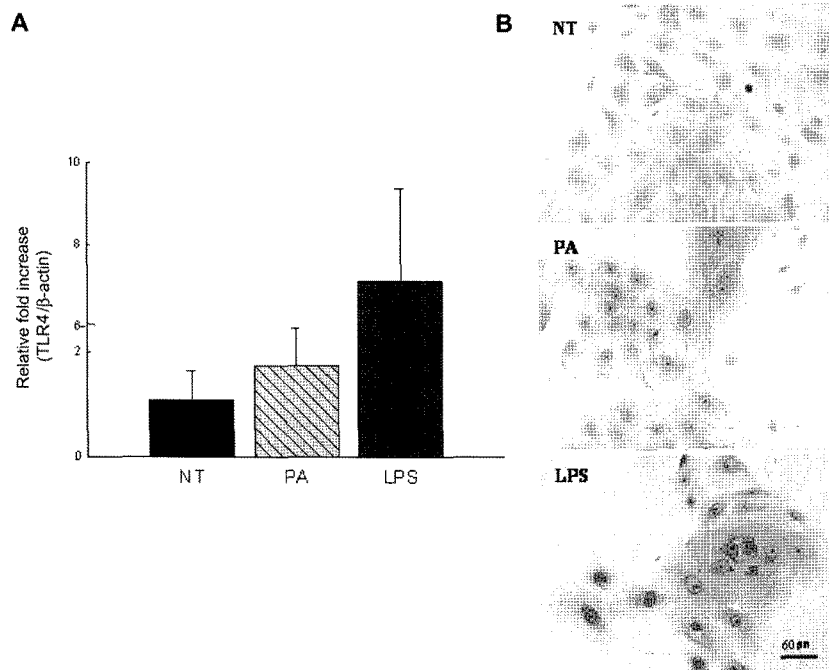


**Fig. 1.** IL-8 mRNA expression induced by *Propionibacterium acnes* in cultured keratinocytes.

The keratinocytes were incubated with heat-killed *P. acnes* at the concentrations of  $5 \times 10^7$  and  $1 \times 10^8$  bacteria/ml for 6 h, respectively. IL-8 mRNA was constitutively expressed in cultured keratinocytes. The level of the IL-8 mRNA expression by *P. acnes* treatment was much higher at the concentration of  $1 \times 10^8$  bacteria/ml, as compared with that at the concentration of  $5 \times 10^7$  bacteria/ml.



**Fig. 2.** Toll-like receptor (TLR) 2 expression induced by *Propionibacterium acnes* in cultured keratinocytes. The keratinocytes were incubated with *P. acnes* at the concentration of  $1 \times 10^8$  bacteria/ml for 6 h. LTA was used as a positive control, and culture medium without any stimulants was used as a negative control. The *P. acnes*-induced TLR2 mRNA expression was significantly increased, 1.8-fold compared with that of the NT cells (A). Moderate cytoplasmic staining for TLR2 was noted on the *P. acnes*-treated keratinocytes (B). \* $p < 0.05$ . Scale bar, 60 μm. LTA, lipoteichoic acid; NT, non-treated; PA, *Propionibacterium acnes*.

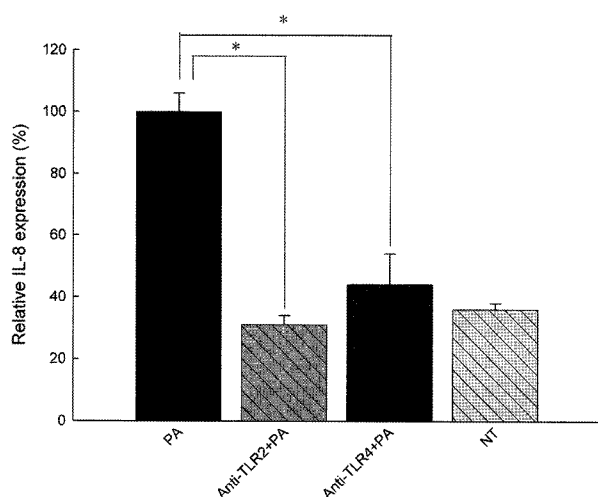


**Fig. 3.** Toll-like receptor (TLR) 4 expression induced by *Propionibacterium acnes* in cultured keratinocytes. The keratinocytes were incubated with heat-killed *P. acnes* at the concentration of  $1 \times 10^8$  bacteria/ml for 6 h. LPS was used as a positive control, and culture medium without any stimulants was used as a negative control. The *P. acnes*-induced TLR4 mRNA expression was increased 1.75-fold compared with that of the NT cells, but the difference was not significant (A). Moderate cytoplasmic staining for TLR4 was noted on the *P. acnes*-treated keratinocytes (B). Scale bar, 60 μm. LPS, lipopolysaccharide; NT, non-treated; PA, *Propionibacterium acnes*.

expression at both concentrations, but it was higher at the  $1 \times 10^8$  bacteria/ml concentration than that at the  $5 \times 10^7$  bacteria/ml concentration. From this result, we decided to choose the concentration of *P. acnes* as  $1 \times 10^8$  bacteria/ml in the next experiments.

#### *P. acnes* Treatment Increased the TLR2 and TLR4 Expressions in the Keratinocytes

To determine whether *P. acnes* treatment induces the mRNA and protein expressions of TLR2 and TLR4 in cultured keratinocytes, the cells were incubated with *P. acnes* at the concentration of  $1 \times 10^8$  bacteria/ml for 6 h, and then the mRNA and protein expressions of TLR2 and TLR4 were analyzed by real-time RT-PCR and immunocytochemistry, respectively. Ten  $\mu\text{g/ml}$  of LTA and 10  $\mu\text{g/ml}$  of LPS were used as positive controls for the TLR2 and TLR4 expressions, respectively. The *P. acnes*-induced TLR2 mRNA expression was significantly increased compared with the negative control ( $p < 0.05$ ) (Fig. 2A). Immunohistochemistry revealed that the *P. acnes*-treated keratinocytes were immunoreactive to anti-TLR2 antibody, as were the LTA-treated keratinocytes (Fig. 2B). The *P. acnes*-induced TLR4 mRNA expression was 1.75-fold increased compared with the negative control, but the difference was not significant (Fig. 3A). Moderate cytoplasmic staining for TLR4 was noted on the *P. acnes*-treated keratinocytes (Fig. 3B). These results demonstrated that *P. acnes* treatment increased both the TLR2 and TLR4 expressions in the cultured keratinocytes.

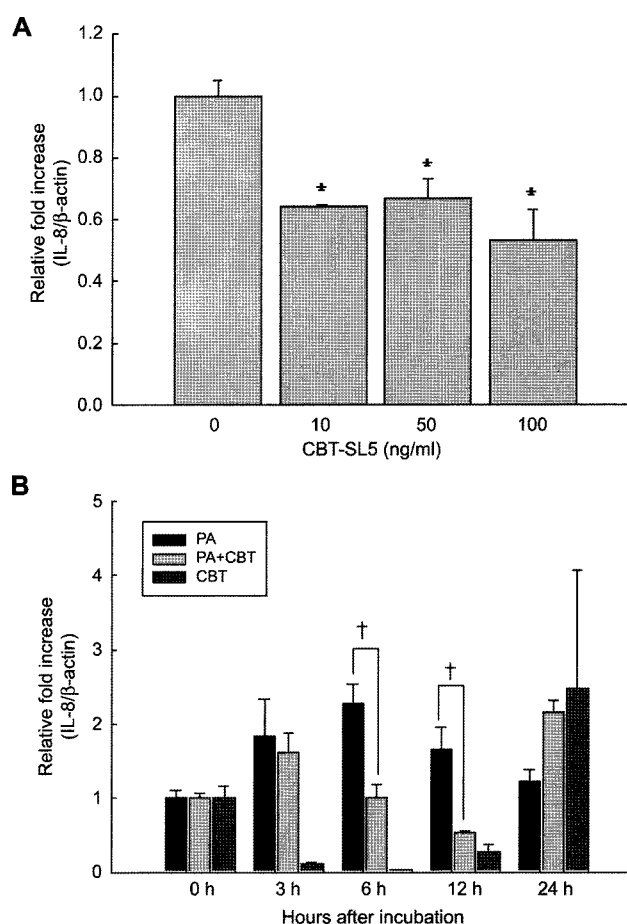


**Fig. 4.** Suppression of the *Propionibacterium acnes*-induced IL-8 mRNA expression in cultured keratinocytes by anti-TLR2 antibody or anti-TLR4 antibody.

The keratinocytes were pre-incubated with anti-TLR2 antibody or anti-TLR4 antibody for 1 h, and then they were stimulated with *P. acnes* at the concentration of  $1 \times 10^8$  bacteria/ml for 3 h. The IL-8 mRNA expression value was normalized with  $\beta$ -actin. Pre-incubation with anti-TLR2 or anti-TLR4 antibody significantly suppressed the IL-8 mRNA expression in the *P. acnes*-treated keratinocytes. \* $p < 0.05$ . NT, non-treated; PA, *P. acnes*.

#### The *P. acnes*-Induced IL-8 mRNA Expression in Keratinocytes was Brought About by TLR2 and TLR4

To determine whether the *P. acnes*-induced IL-8 mRNA expression in keratinocytes was brought about through TLR2 or TLR4, the cultured keratinocytes were pre-incubated either with anti-TLR2 antibody or anti-TLR4 antibody 1 h before *P. acnes* treatment. We then evaluated the IL-8 mRNA expression by real-time RT-PCR. Pre-treatment with anti-TLR2 antibody or anti-TLR4 antibody significantly suppressed the IL-8 mRNA expression in the *P. acnes*-treated keratinocytes ( $p < 0.05$ ). These results



**Fig. 5.** The suppressive effect of CBT-SL5 on the IL-8 mRNA expression induced by *Propionibacterium acnes* in cultured keratinocytes.

CBT-SL5 at the concentrations of 0, 10, 50, and 100 ng/ml was used for the dose-response analysis. One hundred ng/ml of CBT-SL5 was used for the time-response analysis. The levels of the IL-8 mRNA expression were normalized to the  $\beta$ -actin levels and they are presented as relative fold increases, compared with *P. acnes* treatment alone. CBT-SL5 at the concentrations of 10, 50, and 100 ng/ml significantly suppressed the *P. acnes*-induced IL-8 mRNA expression ( $p < 0.05$ ) (A). The hundred ng/ml of CBT-SL5 significantly inhibited the *P. acnes*-induced IL-8 mRNA expression at 6 and 12 h (B). \* $p < 0.05$  when compared with *P. acnes* treatment alone, † $p < 0.05$  when compared with *P. acnes* treatment alone at each time. CBT, treated with CBT-SL5 (100 ng/ml); PA, treated with *P. acnes*; PA+CBT, simultaneously treated with *P. acnes* and CBT-SL5.

showed that the *P. acnes*-induced IL-8 mRNA expression in keratinocytes was brought about by TLR2 and TLR4.

### CBT-SL5 Suppressed the *P. acnes*-Induced IL-8 mRNA Expression in the Keratinocytes

To determine whether CBT-SL5 suppressed the *P. acnes*-induced IL-8 mRNA expression in cultured keratinocytes, various concentrations of CBT-SL5 (0, 10, 50, and 100 ng/ml) were used in this study. The CBT-SL5, at the concentrations of 10, 50, and 100 ng/ml ( $p < 0.05$ ) significantly suppressed the IL-8 mRNA expression of the *P. acnes*-treated keratinocytes, and the CBT-SL5 was most effective at the

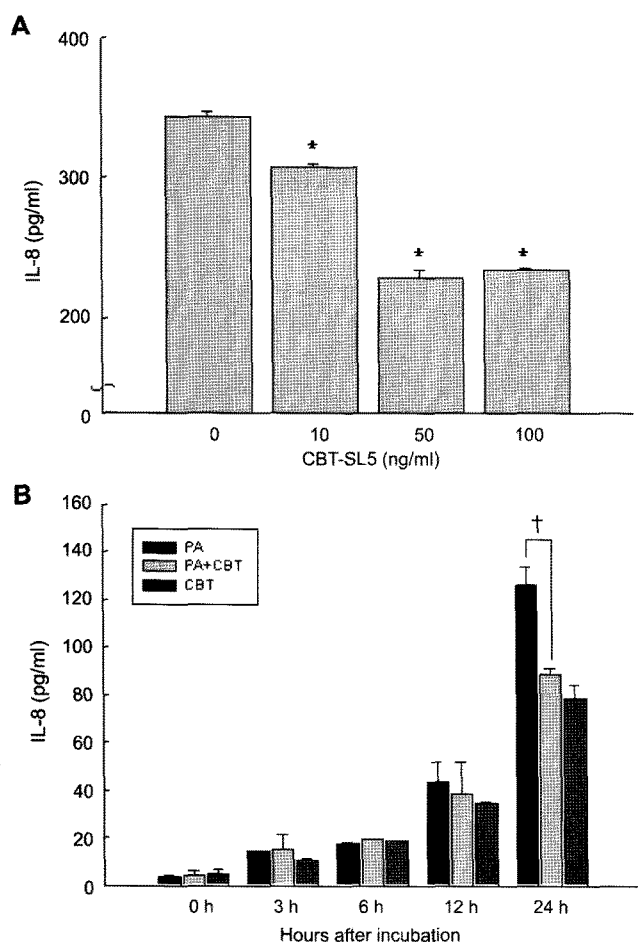
concentration of 100 ng/ml. Thus, 100 ng/ml of CBT-SL5 was subsequently used for the next experiments (Fig. 5A). Next, to determine the time response of the suppressive effect of CBT-SL5 on the *P. acnes*-induced IL-8 mRNA expression, the cultured keratinocytes were treated with *P. acnes* and CBT-SL5 at the concentration of 100 ng/ml for 3, 6, 12, and 24 h. The CBT-SL5 significantly suppressed the *P. acnes*-induced IL-8 mRNA expression at 6 and 12 h, compared with baseline ( $p < 0.05$ ). Unexpectedly, the treatment with the CBT-SL5 augmented rather than suppressed the IL-8 mRNA expression at 24 h (Fig. 5B).

### CBT-SL5 Suppressed *P. acnes*-Induced IL-8 Protein Production in the Cultured Keratinocytes

To further determine whether CBT-SL5 suppressed *P. acnes*-induced IL-8 production in the cultured keratinocytes, we measured the IL-8 protein by performing ELISA. As shown in Fig. 6, CBT-SL5 significantly suppressed *P. acnes*-induced IL-8 production in the cultured keratinocytes at the concentrations of 10, 50, and 100 ng/ml ( $p < 0.05$ ). For the time-response results, 100 ng/ml of CBT-SL5 significantly reduced the IL-8 secretion induced by *P. acnes* at 24 h only ( $p < 0.05$ ).

### CBT-SL5 Suppressed *P. acnes*-Induced NF- $\kappa$ B Translocation

It has been known that NF- $\kappa$ B plays a key role in the regulation of several proinflammatory cytokine genes, including IL-8, and its translocation from the cytoplasm to the nucleus activates the processes of gene expression and protein synthesis [1, 8, 14]. To determine whether CBT-SL5 can regulate the signal of TLR-to-NF- $\kappa$ B, we performed immunofluorescent staining against the NF- $\kappa$ B p65. The PBS-treated keratinocytes primarily demonstrated cytoplasmic NF- $\kappa$ B p65 staining (Fig. 7A). LTA treatment, as a positive control, in the keratinocytes caused NF- $\kappa$ B p65 translocation to the nucleus (Fig. 7B). Likewise, *P. acnes* treatment also provoked NF- $\kappa$ B p65 translocation (Fig. 7C), but this was prevented by simultaneous incubation with CBT-SL5 (Fig. 7D). Incubation with CBT-SL5 alone had no effect on p65 translocation in the keratinocytes (Fig. 7E).

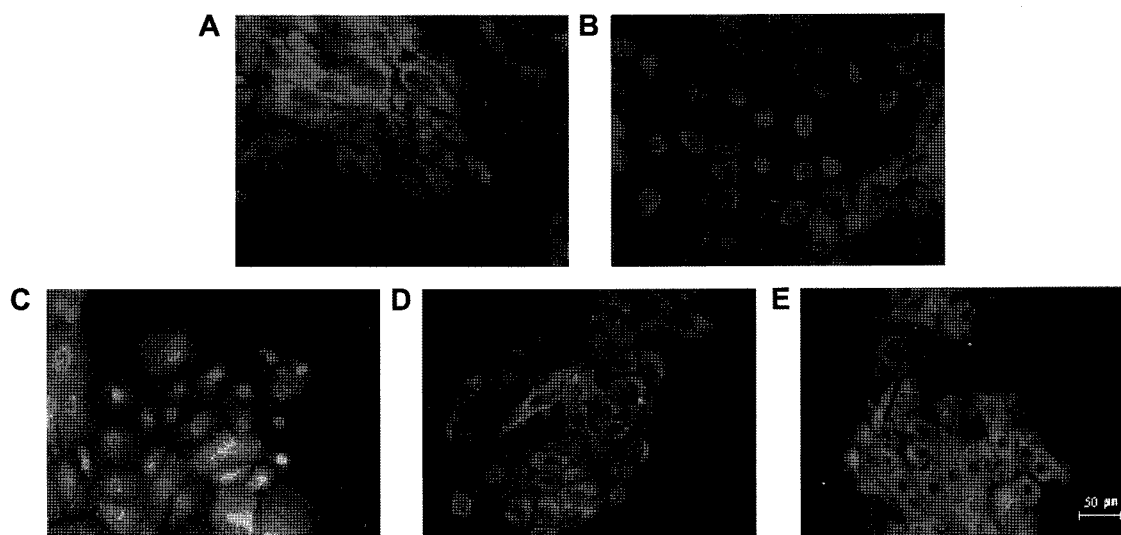


**Fig. 6.** The suppressive effect of CBT-SL5 on the IL-8 protein production induced by *Propionibacterium acnes* in cultured keratinocytes.

CBT-SL5 at the concentrations of 10, 50, and 100 ng/ml was used for the dose-response analysis. The amount of IL-8 protein in the supernatant was measured by ELISA. CBT-SL5 at the concentrations of 10, 50, and 100 ng/ml significantly inhibited the *P. acnes*-induced IL-8 protein secretion ( $p < 0.05$ ) (A). One hundred ng/ml of CBT-SL5 significantly inhibited the *P. acnes*-induced IL-8 protein secretion at 24 h ( $p < 0.05$ ) (B). \* $p < 0.05$  when compared with *P. acnes* treatment alone, † $p < 0.05$  when compared with *P. acnes* treatment alone at each time. CBT, treated with CBT-SL5 (100 ng/ml); PA, treated with *P. acnes*; PA+CBT, simultaneously treated with *P. acnes* and CBT-SL5.

## DISCUSSION

There have been several reports that bacteriocins have antimicrobial activities against *P. acnes* [2, 21]. Oh *et al.* [22] demonstrated that the bacteriocin produced by *Lactococcus* sp. HY 449 caused bacterial lysis and it inhibited the growth of *P. acnes*. Bove *et al.* [2] also demonstrated that the bacteriocin produced by *Streptococcus salivarius* inhibited the growth of *P. acnes*. Moreover, in our previous study, we demonstrated that the CBT-SL5 produced by *E. faecalis* had a killing effect against *P. acnes* ATCC 29399



**Fig. 7.** The inhibitory effect of CBT-SL5 on *Propionibacterium acnes*-induced NF- $\kappa$ B translocation to the nucleus in the cultured keratinocytes.

Incubation with PBS, which was used as a negative control, revealed cytoplasmic NF- $\kappa$ B p65 staining (A). Incubation with lipoteichoic acid, which was used as a positive control, for 30 min caused p65 translocation to the nucleus (B). Similarly, *P. acnes* treatment caused p65 translocation (C), whereas simultaneous incubation with CBT-SL5 inhibited *P. acnes*-induced p65 translocation to the nucleus (D). CBT-SL5 alone had no effect on the p65 translocation (E).

and KCTC 3314. In addition, the same study also revealed that CBT-SL5 significantly reduced the mild to moderate inflammatory lesions (pustules) compared with the placebo group (unpublished observation). From our previous results, it might be assumed that CBT-SL5 can reduce the chemoattractive activities of neutrophils, which are mainly due to the IL-8 secreted by immune cells.

In this study, our results showed that CBT-SL5 reduced the IL-8 mRNA expression and protein secretion in *P. acnes*-treated keratinocytes. In our culture condition, CBT-SL5 at concentrations less than 100 ng/ml downregulated the IL-8 mRNA expression and protein secretion in the cultured keratinocytes that were treated by *P. acnes*. CBT-SL5 also downregulated the IL-8 mRNA expression at 6 and 12 h, and it also downregulated IL-8 protein production at 24 h in the cultured keratinocytes that were treated by *P. acnes*. These results demonstrated that CBT-SL5 can suppress the IL-8 expression induced by *P. acnes* in keratinocytes.

There have been several similar studies that have shown the regulatory effect of LL-37 on the IL-8 production, and a low concentration ( $\leq 10$   $\mu$ g/ml) of LL-37 could also reduce the IL-8 and MCP-1 production in LPS-treated monocytes. However, at the higher concentration ( $\geq 10$  mg/ml), LL-37 induced rather than reduced IL-8 and MCP-1 production [24]. In addition, Nagaoka *et al.* [19] demonstrated that a cationic antibacterial polypeptide of 11 kDa (CAP11), which is an AMP isolated from guinea pig neutrophils, inhibited the production of IL-1 $\beta$ , TNF- $\alpha$ , and IL-8 in LPS-treated human PBMCs. McInturff *et al.* [18] recently demonstrated

that granulysin and synthetic granulysin-derived peptides could downregulate the production of inflammatory cytokines and chemokines such as IL-12p40, IP-10, MCP-1, and IL-8 in *P. acnes*-stimulated monocytes.

The precise mechanism of the anti-inflammatory effect of CBT-SL5 on the *P. acnes*-induced inflammation in keratinocytes is still unclear. Two potential mechanisms of the anti-inflammatory actions of AMPs have been recently proposed by Marta Guarna *et al.* [17]: one is a direct anti-inflammatory effect of CBT-SL5 on the signal cascades in host cells, and the other is a neutralization of an inflammatory bacterial factor by interaction with various pathogen-associated molecular patterns (PAMPs). Prior to gaining insight on the crucial mechanism of CBT-SL5, we need to know more about the signal pathway for the *P. acnes*-induced inflammation in keratinocytes.

From other previous studies, it has been known that *P. acnes* induced inflammation in keratinocytes *via* the TLR signal pathway [10, 13]. Although the immunogenic components of *P. acnes* are still unclear, bacterial cell wall components such as PGN and LTA have been documented as possible candidates [13, 31]. One study demonstrated that treatment with the cell wall components of *P. acnes* induced both TLR2 and TLR4 expressions during the early hours of incubation in human keratinocytes [13]. Our results also demonstrated that heat-killed *P. acnes* augmented the TLR2 and TLR4 expressions in human keratinocytes. Generally, activation of the TLR2 and TLR4 signaling pathways goes downstream, and it finally activates NF- $\kappa$ B; this causes regulation of the genes that are involved in

inflammation [28]. In this study, it was shown that the *P. acnes*-induced IL-8 mRNA expression was significantly suppressed by anti-TLR2 or anti-TLR4 neutralizing antibodies. These results are in agreement with results by Nagy *et al.* [20], in which the *P. acnes*-induced secretion of hBD2 and IL-8 in keratinocytes was both TLR2- and TLR4-dependent. It means that the *P. acnes*-induced inflammation was mediated by the TLR-to-NF- $\kappa$ B signal cascade in keratinocytes. According to our immunofluorescent staining results, CBT-SL5 inhibited *P. acnes*-activated NF- $\kappa$ B translocation to the nucleus. Thus, these results suggested that CBT-SL5 could possibly directly inhibit the signal transduction at the upstream level of NF- $\kappa$ B.

On the other hand, in this study, CBT-SL5 suppressed the IL-8 expression in keratinocytes when *P. acnes* and CBT-SL5 were administered simultaneously. From this result, it might be assumed that CBT-SL5 may be interacting with *P. acnes* and binding up the TLR ligands that are present in *P. acnes*, and so this prevents some of these components from activating TLRs. Although we could not confirm this possibility in this study, some previous reports have shown that AMPs could directly bind with PAMPs and cause suppression of cytokine secretion. Scott *et al.* [25] demonstrated that CEME and its derivatives bound to LTA with high affinity, and this blocked LTA-induced production of TNF- $\alpha$  in the murine macrophage cell line RAW 264.7. Additionally, that study also demonstrated that CEME and its derivatives could block the interaction of LPS with LBP and the production of TNF- $\alpha$  in the same cell line [27]. The precise anti-inflammatory mechanism of CBT-SL5 needs further investigation.

In conclusion, based on our results, we demonstrated that CBT-SL5 suppressed the *P. acnes*-induced IL-8 expression in keratinocytes. Therefore, CBT-SL5 may be a novel anti-inflammatory modality for treating acne.

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