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Inhibitory Effect of Chlorophyllin on the *Propionibacterium acnes*-Induced Chemokine Expression

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Chlorophyllin (CHL), a chlorophyll-derivative, exhibits several beneficial properties, including antibacterial, antioxidant, and anticancer activities. However, its antibacterial and anti-inflammatory activities against *Propionibacterium acnes* have not been described. The antibacterial activity of this compound was evaluated *in vitro* using the broth microdilution method. CHL had an inhibitory effect on the growth of *P. acnes* (MIC = 100 μM). In a real-time reverse transcription-polymerase chain reaction and an enzyme-linked immunosorbent assay, CHL significantly decreased interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) production in a dose-dependent manner, decreasing both mRNA and protein levels for these chemokines in THP-1 cells indicating the anti-inflammatory effects of it. To investigate the molecular mechanisms underlying the anti-inflammatory properties of CHL in THP-1 cells stimulated by *P. acnes*, we used western blotting to analyze the effect of CHL on activation of the nuclear factor (NF)-κB. CHL inhibited *P. acnes*-induced IL-8 and MCP-1 production *via* blockade of NF-κB activation in THP-1 cells. Therefore, based on these results, we suggest that CHL is a useful agent to control the growth of *P. acnes* involved in acne inflammation and prevent acne.

Keywords: chlorophyllin, interleukin-8, monocyte chemotactic protein 1, *Propionibacterium acnes*, THP-1 cells

Introduction

Acne vulgaris is one of the most common skin diseases, and is observed in approximately 80% of young adults (Johnson *et al*., 1984). Acne is affected by several factors, specifically hormonal imbalances, bacterial infections, stress, food, or cosmetic application (Burkhart *et al*., 1999). Normal skin commensals usually consist of the aerobic coccus *Staphylococcus epidermidis*, the yeasts *Malassezia furfur* and the anaerobic diphtheroid *Propionibacterium acnes* (Holland *et al*., 2008). Among them, *P. acnes* has been implicated in contributing to the inflammatory response of acne (Webster *et al*., 1978). It acts an immunostimulator that can produce a variety of enzymes and biologically active molecules, such as lipases, proteases, hyaluronidases, and chemotactic factors, which are involved in the development of inflammatory acne. The main components of the pilosebaceous unit on the skin can be activated by *P. acnes*, leading to the production of pro-inflammatory cytokines (Leeming *et al*., 1985; Vowels *et al*., 1995).

Chemokines directly attract and activate specific types of cells *in vitro* and are divided mainly into two major subfamilies, C-X-C and C-C chemokines (van Deventer, 1997). Interleukin-8 (IL-8) is a CXC-type chemokine that binds to the cellular seven transmembrane domain G protein-coupled receptors known as CXCR1 and CXCR2 (Yoshimura *et al*., 1987; Hoch *et al*., 1996). Monocyte chemoattractant protein-1 (MCP-1), a chemokine (C-C motif) ligand 2 (CCL2), attracts monocytes, memory T cells, and dendritic cells to the sites of inflammation produced by either tissue injury or infection (Rollins, 1996). In most cell types, transcriptional control of IL-8 or MCP-1 expression appears to be the pivotal regulatory mechanism.

As therapeutic agents for acne, antibiotics are normally used to inhibit inflammation or kill bacteria (Guin *et al*., 1979). Triclosan, benzoyl peroxide, azelaic acid, retinoid, tetracycline, erythromycin, macrolide, and clindamycin are among these antibiotics (Breathnach *et al*., 1984; Zouboulis and Piquero-Martin, 2003). On the other hand, these antibiotics also produce side effects including xerosis cutis, skin irritation, appearances of resistant bacteria, organ damage, and immunehypersensitivity if taken for a long time (Leyden *et al*., 2007; Patel *et al*., 2010). Therefore, many researchers have tried to develop therapeutic agents for acne with no side effects but high antibacterial activity (Nam *et al*., 2003; Kang *et al*., 2009; Niyomkam *et al*., 2010).

Chlorophyllin (CHL) is an artificial water-soluble form of chlorophyll. Chlorophyll, the photosynthetic green pigment, is present in food materials of plant origin as well as nutritional supplements including Spirulina and extracts from *Chlorella vulgaris* (Kay, 1991) that are credited with several beneficial properties (Negishi *et al*., 1997; Dashwood *et al*., 1998). It has been reported that CHL exhibits antimicrobial activity against several microorganisms (Nevin and Bibby, 1954; Benati *et al*., 2009). CHL has several additional beneficial functions as an antioxidant and anticarcinogenic effects

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(Kumar *et al*., 2001, 2004; Castro *et al*., 2009). However, these studies did not report the effects of CHL on the inflammatory reaction of *P. acnes*. Based on the known beneficial effects of CHL, we investigated the possibility that it may be effective as an acne treatment.

In this study, for antibacterial and anti-inflammatory activity of CHL against *P. acnes*, we examined the effects of CHL on the growth of *P. acnes* as well as the production of inflammatory mediators, IL-8 and MCP-1, by THP-1 human monocytic cells stimulated with *P. acnes.*

Materials and Methods

Materials

Chlorophyllin sodium copper salt (CHL) was purchased from Sigma (USA).

Bacterial culture

Propionibacterium acnes ATCC 11828 was grown in Actinomyces broth (Difco, USA) under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) at 37°C for 24 h. *Staphylococcus epidermidis* ATCC 12228 and *Staphylococcus aureus* ATCC 19213 were grown in brain heart infusion broth (BHI broth; Difco) under aerobic conditions at 37°C for 16 h. Bacterial cultures in the logarithmic growth phase were used in all experiments. Before being used in each experiment, they were propagated twice in the appropriate broth.

Cell culture

The human monocytic THP-1 cell line (KCLB 40202) was grown in RPMI 1640 (WelGENE, Korea) supplemented with 10% fetal bovine serum (Gibco, UK), L-glutamine (300 mg/L), 25 mM HEPES, and sodium bicarbonate (2,000 mg/L) at 37°C, in a humidified atmosphere containing 5% CO_2.

Antibacterial assay

Antibacterial activity of CHL was carried out using a broth microdilution method following the Clinical and Laboratory Standards Institute guidelines (Citron and Hecht, 2003; Jorgensen and Turnidge, 2003). The logarithmic phase cultures of the bacteria were added to the culture medium containing a series of CHL dilutions in the wells of microtiter plates, and their growth was assessed after a period of incubation. The inoculum size was controlled by measuring the optical density at 600 nm and extrapolating the colony forming unit (CFU)/ml using preset standard curves. Successive two-fold dilutions of CHL were prepared in a 100 μl volume, and 100 μl each of bacteria was added to the prepared plates. The final inoculum concentrations of *P. acnes*, *S. epidermidis*, and *S. aureus* were 1×10^6, 5×10^5, and 5×10^5 CFU/ml, respectively. The wells of the plates included one growth and one sterile control. After incubation under anaerobic or aerobic conditions at 37°C for 24 h, the level of microbial growth was measured using a microplate reader (Molecular Devices, USA) at 600 nm. The minimum inhibitory concentration (MIC) was defined as the lowest dilution of CHL at which no growth was detected.

Infection protocol

Bacteria were washed twice with phosphate-buffered saline (PBS) and once with complete RPMI medium. After resuspension in complete RPMI medium, the optical density of the bacterial suspension was measured at 600 nm, and further diluted to an optical density of 0.5 which corresponded to 5×10^8 CFU/ml. For quantitative reverse transcription and polymerase chain reaction (qRT-PCR), 6-well plates were seeded with 3×10^6 THP-1 cells, treated with concentrations of CHL (25 or 100 μM) and incubated with *P. acnes* [multiplicity of infection (MOI) of 1:100] for 6 h. For enzyme-linked immunosorbent assays (ELISA), the THP-1 cells were plated at 5×10^5 cells/well into 24-well plates, treated with various concentrations of CHL (25–200 μM) and then stimulated with *P. acnes* (MOI of 1:100) for 24 h. For Western blots, THP-1 cells (6×10^6 cells/well, 10 ml) were plated onto a 100 mm tissue culture dish, treated with 25 or 100 μM CHL and then incubated with *P. acnes* (MOI of 1:100) for 1, 3, and 6 h.

RNA isolation and qRT-PCR

The total RNA was isolated from the THP-1 cells according to the manufacturer's specifications using the RNAeasy mini kit (QIAGEN, Germany). The total RNA (3 μg) was reverse transcribed with M-MLV cDNA synthesis system (Invitrogen, USA), according to the manufacturer's instructions. Relative quantification of gene expression by real-time PCR was performed in the Rotor-Gene 6000 system (QIAGEN). Real-time PCR reactions were carried out using SYBR® Premix Ex Taq II (TaKaRa, Japan), QuantiTect Primer Assay (QIAGEN; IL-8 QT00000322 and MCP-1 QT00212730), and 20 ng of cDNA sample, in a total volume of 20 μl. The cycling conditions were 1 cycle of denaturation at 95°C /30 sec, followed by 40 two-segment cycles of amplification (95°C/5 sec, 60°C/30 sec) where the fluorescence was automatically measured during PCR. The $\Delta\Delta C_T$ method for relative quantitation of gene expression was used to determine mRNA expression levels. The relative level of each analyte over internal standard (GAPDH) was calculated by using the equation $2^{-\Delta\Delta CT}$ where $\Delta C_T = C_T$ (analyte) – C_T (internal standard), and then compared between different groups or treatments. All samples were amplified in triplicates and the mean was used for further analysis.

Cytokine assay

The THP-1 culture supernatants were collected, clarified, and levels of IL-8 and MCP-1 were quantified using a commercial ELISA kit (R&D Systems, USA) according to the manufacturer's directions.

Western blot

THP-1 cells were harvested and lysed with mammalian protein extraction reagent (M-PER) (Thermo Scientific, USA) containing protease inhibitor cocktail (Roche Molecular Diagnostics, Germany). The whole-cell lysate (20 μg) was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane (Pall Life Sciences, UK). The membrane was

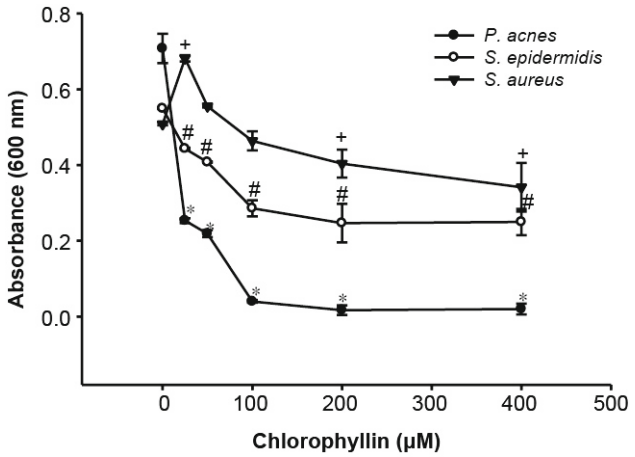

Fig. 1. Determination of the antibacterial activity of CHL for acne-inducing bacteria. Serial doses of CHL were added to the bacterial cultures in 96-well plates and incubated under anaerobic or aerobic conditions for 24 h. The growth of bacteria was determined by measuring the optical density of the cultures at 600 nm. The values are reported as the Mean ±SD of three independent experiments. **P*<0.05, CHL-treated group vs. *P. acnes* control; # *P*<0.05, CHL-treated group vs. *S. epidermidis* control; + *P*<0.05, CHL-treated group vs. *S. aureus* control.

blocked with 5% skim milk in 1× TBST (0.01 M Tris; pH 7.6, 0.1 M NaCl, and 0.1% Tween 20) for 2 h at room temperature with shaking and incubated with rabbit polyclonal antibodies against phosphorylated IκB-α (p-IκB-α), phosphorylated p65 (p-p65), or β-actin (1:1000, Cell Signaling Technology, USA), followed by HRP-conjugated secondary antibody. The membranes were washed and then incubated in enhanced chemiluminescence substrate (Amersham Biosciences, USA) for 5 min before X-ray film exposure.

Cytotoxicity assay

General viability of cultured cells was determined by the reduction of WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt] (Cay man, USA) to a highly water-soluble formazan dye (Ishiyama *et al.*, 1997). This assay was performed after the incubation of THP-1 cells treated with various concentrations of CHL (25–400 μM) for 24 h at 37°C in humidified atmosphere containing 5% CO_2. The control group cultures were left untreated. Ten microliters of WST-8 solution was added to each well. Cells were incubated at 37°C for 4 h. The absorbance of each well was measured at 450 nm using an automatic microplate reader.

Statistics

Each experiment was carried out in triplicate, and the mean value was analyzed further. Statistical analysis was carried out using SPSS version 17.0 (Statistical packages for Social Science version 17.0; SPSS Inc., USA). A Mann-Whitney test was used to identify any statistically significant differences in the experiments.

Results

Determination of the antibacterial activity of CHL for acne-inducing bacteria

The antibacterial activities of CHL against *P. acnes*, *S. epidermidis*, and *S. aureus* were determined using a broth microdilution method. As shown in Fig. 1, the growth of *P. acnes* was inhibited completely by CHL (MIC=100 μM). However, the growth of *S. epidermidis* and *S. aureus* were not inhibited by a high concentration of CHL. CHL (400 μM) exerted 55% and 33% growth inhibition of *S. epidermidis* and *S. aureus*, respectively. For this reason, *P. acnes* was selected and used in further experiments.

Effect of CHL on mRNA and protein synthesis of IL-8 and MCP-1

THP-1 cells were stimulated with *P. acnes* in the presence or absence of CHL for 6 h, after which IL-8 and MCP-1 mRNA expressed by the cells were quantified by real-time RT-PCR. CHL suppressed the *P. acnes*-induced expression of the IL-8 and MCP-1 genes in a dose-dependent manner (25 μM, 30.2±2.4%; 100 μM, 77.1±1.4% inhibition in IL-8; 25 μM, 84.9±0.7%; 100 μM, 98.1±0.0% inhibition in MCP-1) (*P*<0.05) (Fig. 2). IL-8 and MCP-1 secretion were measured

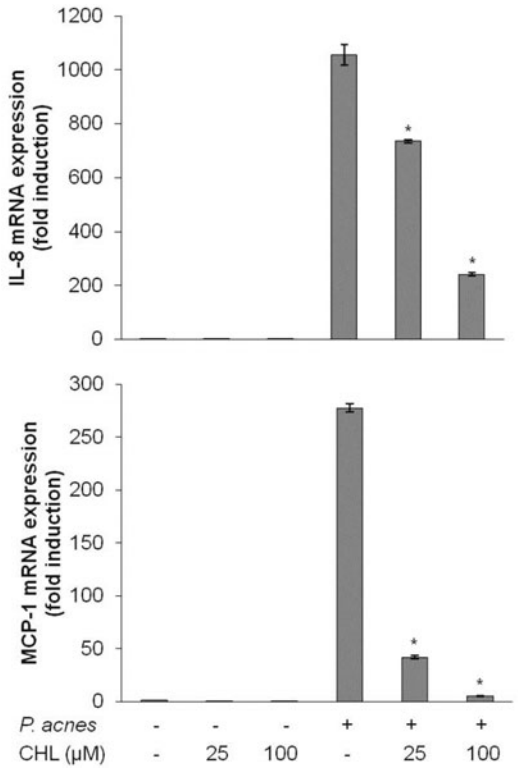

Fig. 2. Effect of CHL on IL-8 and MCP-1 mRNA expression in THP-1 cells stimulated with *P. acnes*. THP-1 cells were treated with the indicated concentrations of CHL before being stimulated with *P. acnes* (1:100) for 6 h. The total RNA was isolated, reverse transcribed and the cDNA samples were amplified using real-time quantitative PCR and SYBR Green detection as described in 'Materials and Methods'. The values are reported as the Mean±SD of three independent experiments. **P*<0.05, cells stimulated with *P. acnes* in the presence of CHL vs. *P. acnes* control.

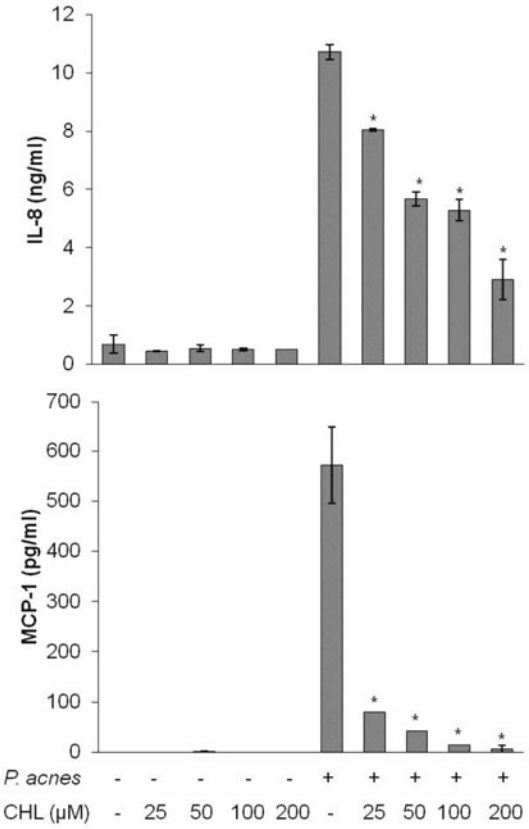

Fig. 3. Effect of CHL on IL-8 and MCP-1 production in THP-1 cells stimulated with *P. acnes*. THP-1 cells were treated with the indicated concentrations of CHL before being infected with *P. acnes* (1:100) for 24 h. The secretion of IL-8 and MCP-1 was measured by ELISA, as described in 'Materials and Methods'. The values are reported as the mean±SD of three independent experiments. **P*<0.05, cells stimulated with *P. acnes* in the presence of CHL vs. *P. acnes* control.

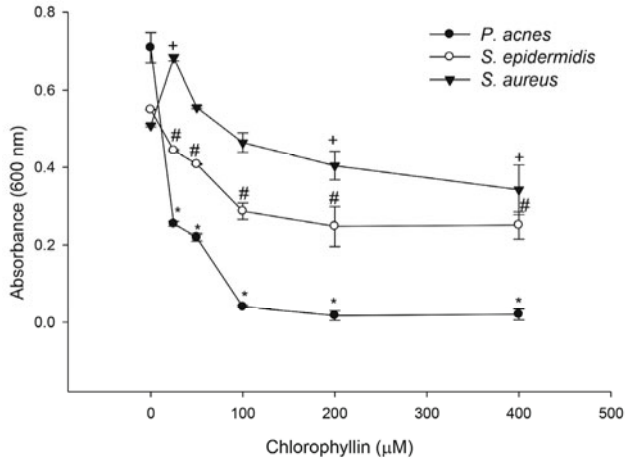

Fig. 4. Effect of CHL on *P. acnes*-induced NF-κB activation in THP-1 cells. THP-1 cells were incubated in the presence or absence of *P. acnes* along with 100 μM of CHL for the indicated times. At each time, whole-cell lysates were prepared and subjected to Western blotting using antibodies specific to p-IκB-α, p-p65, or β-actin as described in 'Materials and Methods'. Western blot analysis experiments performed in triplicate.

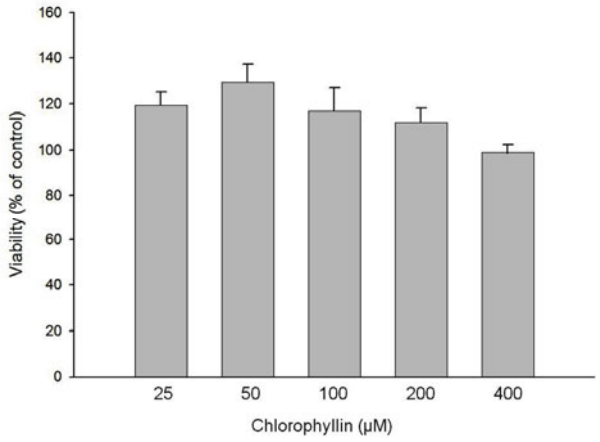

Fig. 5. Cytotoxicity of CHL on THP-1 cells. The THP-1 cells were treated with CHL (25–400 μM) for 24 h. Untreated cells were used as a control. The cell viability was assessed using a WST-8 assay. The values are reported as the Mean±SD of three independent experiments.

by ELISA to confirm the association of mRNA expression with protein production. Stimulation of THP-1 cells with *P. acnes* during 24 h induced the secretion of both cytokines. Treatment with CHL also blocked the secretion of IL-8 and MCP-1 induced by *P. acnes* in a dose-dependent manner (25 μM, 24.8±1.4%; 50 μM, 46.9±3.5%; 100 μM, 50.6±4.6%; 200 μM, 72.7±7.1% inhibition in IL-8; 25 μM, 85.8±1.9%; 50 μM, 92.5±1.0%; 100 μM, 97.4±0.3%; 200 μM, 98.8±1.4% inhibition in MCP-1) (*P*<0.05) (Fig. 3).

Effect of CHL on *P. acnes*-induced nuclear factor (NF)-κB activation in THP-1 cells

To investigate the molecular mechanisms underlying the anti-inflammatory properties of CHL in THP-1 cells stimulated by *P. acnes*, THP-1 cells were stimulated by *P. acnes* in the presence of CHL. Subsequently, NF-κB signal pathway was determined by western blot analysis. Infection with *P. acnes* led to phosphorylation of IκB-α and p65 proteins in THP-1 cells. However, CHL inhibited *P. acnes*-induced p-IκB-α and p-p65 expression in a time-dependent manner (Fig. 4).

Cytotoxicity of CHL

To determine whether CHL induced cytotoxicity, we performed WST-8 assays in THP-1 cells. As shown in Fig. 5, CHL showed no cytotoxic effects at all tested concentrations (25–400 μM).

Discussion

CHL, the sodium-copper salt and the water-soluble analogue of the ubiquitous green pigment chlorophyll, is attributed with several beneficial properties. Several studies reported CHL antimicrobial effects (Nevin and Bibby, 1954; Benati *et al*., 2009). Many authors reported that CHL has a variety of biological effects ranging from anti-oxidant to anticancer effects (Kumar *et al*., 2001, 2004; Castro *et al*.,

2009). The mechanisms responsible, however, are not fully understood. In the biologically complex mammalian system, several mechanisms including the antioxidant property of CHL and many other cellular defense pathways can lead to protective effects *in situ* (Kumar *et al.*, 1999). Some suggestions pertain to direct effects on genotoxicity.

Earlier studies have shown that CHL has the ability to alter the oxidation-reduction potential (Eh) of a synthetic medium as much as 100 mV in favor of oxidation. This suggests that alteration of Eh may be the mechanism by which CHL exerts its inhibitory capacity (Nevin and Bibby, 1954). It has been reported that the growth of *S. aureus* is strongly inhibited by very high concentrations of CHL (4 mg/ml) (Nevin and Bibby, 1954). This suggests that the inhibition of susceptible bacteria by CHL may be the result of its effect in poising Eh at unfavorable potentials for the initiation of growth. However, in the present study, a fairly high concentration of CHL (400 μM) was determined to exert no apparent inhibitory effects on the growth of *S. epidermidis* and *S. aureus.* This shows that CHL has a bacteriostatic effect rather than a bactericidal effect.

However, CHL readily inhibited a standard laboratory strain of *P. acnes* in our study. CHL is a semi-synthetic porphyrin obtained from chlorophyll. Porphyrin molecules are of interest due to their antimicrobial ability. When the molecules are activated by visible light, they generate reactive oxygen species that are cytotoxic to most live cells (Romanova *et al.*, 2003). When these short-life free radicals are in close proximity to the cell surface, they can trigger extensive cell damage, resulting in cell death. In addition, it is reported that *P. acnes* produces porphyrins which absorb light energy (Melø, 1987). Irradiation of *P. acnes* with blue visible light leads to photoexcitation of bacterial porphyrins, singlet oxygen production and eventually bacterial destruction. A possible antibacterial action mechanism of CHL is a reprising of the Eh in such a manner as to inactivate certain metabolic pathways or generate free radicals. Additionally, CHL showed capacity to inhibit IL-8 and MCP-1 production in a dose-dependent manner, decreasing both mRNA and protein levels for these chemokines in THP-1 cells. Therefore, CHL could provide a safe two-fold benefit to acne individuals by inhibiting proliferation of bacterium and reversing the bacterial-induced inflammation.

P. acnes causes an inflammatory acne that is characterized by massive neutrophil infiltration. *P. acnes* secretes lipase and degrades sebum oils into free fatty acids, which are potent acne stimuli. This bacterium also secretes leukocyte chemotatic factors, infiltrating leukocytes in the hair follicle. The interaction between *P. acnes* and infiltrated monocytes and lymphocytes may also play an important role in the pathogenesis of inflammatory acne. IL-8 or MCP-1 is a potent proinflammatory chemotactic factor that directs the migration of monocytes to the site of an infection (Hoch *et al.*, 1996; Rollins, 1996; Baggiolini *et al.*, 1997). IL-8 is thought to play an important role in the pathophysiology of *P. acnes*, although the mechanisms by which *P. acnes* up-regulates the release of IL-8 from target cells is not well understood. The present study showed that IL-8 and MCP-1 mRNA in THP-1 cells was up-regulated by *P. acnes* infection. In addition, *P. acnes* strongly stimulated the production of IL-8 and MCP-1. These results are consistent with the studies demonstrating that *P. acnes* stimulates the production of IL-8 by human monocytic THP-1 cells (Vowels *et al.*, 1995).

Transcriptional control of IL-8 expression appears to be the pivotal regulatory mechanism in most cell types. NF-κB has been as the transcription factor that is involved in the positive regulation of the IL-8 genes (Mukaida *et al.*, 1990, 1994; Yasumoto *et al.*, 1992). To the present study, we investigated the molecular basis for the inhibition of IL-8 and MCP-1 production by CHL, by evaluating the extent to which this molecule interfered with signaling pathways activated when THP-1 cells are stimulated with *P. acnes*. We first confirmed that the activation of NF-κB signaling pathway by *P. acnes* led to phosphorylation of IκB and p65 in THP-1 cells. In unstressed cells, the nuclear localization sequence of NF-κB is bound to inhibitory IκB proteins in the cytosol and these inhibitory proteins inhibit the dimerization of p50 to p65. However, infection of *P. acnes* activates IκB-α kinase, which results in phosphorylation of IκB and p65 proteins. After treatment of CHL, p-IκB-α and p-p65 proteins were down-regulated by blocking the NF-κB pathway. This phosphorylation initiates ubiquitination and subsequent IκB degradation *via* the proteasome. Degradation of IκB removes the inhibition and releases NF-κB complexes so that dimerization and nuclear translocation can occur (DiDonato *et al.*, 1997). These results are consistent with those obtained for the inhibition of IL-8 and MCP-1 mRNA. Our results indicate that CHL inhibits IL-8 and MCP-1 production through blockade of NF-κB activation in THP-1 cells.

In conclusion, the present study showed a previously undescribed anti-inflammatory property of CHL, which involves inhibition of MCP-1 and IL-8. CHL can inhibit the growth of *P. acnes* as well as IL-8 and MCP-1 production in THP-1 human monocytic cells induced by *P. acnes* through blockade of NF-κB activation. This suggests that CHL is a good candidate for controlling acne disease. Further *in vivo* studies will be necessary to evaluate the efficacy and safety for use.

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