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Protective effect of melittin against inflammation and apoptosis on *Propionibacterium acnes*-induced human THP-1 monocytic cell

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ABSTRACT

Melittin is a cationic, hemolytic peptide that is the main toxic component in the venom of the honey bee (*Apis mellifera*). It has been used in treatment of various chronic inflammatory diseases. However, the cellular mechanism and the anti-apoptotic effect of melittin in *Propionibactierium acnes* (*P. acnes*)-induced THP-1 cells have not been explored. In the present study, we investigated the anti-inflammatory and anti-apoptotic mechanism by examining the effect of melittin on *P. acnes*-induced THP-1 monocytic cells. THP-1 monocytic cells were stimulated by heat-killed *P. acnes* in the presence of melittin. The expression levels of pro-inflammatory cytokines, NF-κB signaling, caspase family, and PARP signaling were measured by ELISA or Western blot analysis. The number of apoptotic cells and changes of cell morphology were examined using fluorescence microscopy and flow cytometry. Heat-killed *P. acnes* increased the secretion of pro-inflammatory cytokines and cleavage of caspase-3 and -8 in heat-killed *P. acnes*-induced THP-1 cells. However, treatment with melittin inhibited the pro-inflammatory cytokines and cleavage of the caspase-3 and -8. Moreover, the cleaved PARP appeared after 8 h of heat-killed *P. acnes* treatment and its cleavage was reduced by melittin treatment. These results demonstrate that 1.0×10^7 CFU/ml of heat-killed *P. acnes* induces THP-1 cell apoptosis and secretion of inflammatory cytokines. Also, administration of melittin significantly decreases the expression of various inflammatory cytokines in heat-killed *P. acnes*-treated THP-1 monocytic cells. In particular, melittin exerts anti-apoptotic effects against 1.0×10^7 CFU/ml of heat-killed *P. acnes* injury to THP-1 cells.

1. Introduction

Acne vulgaris is a chronic inflammatory disorder of the sebaceous follicles and is the most common skin disease, estimated to affect up to 80% of individuals at some point between the ages of 11 and 30 years (Grange et al., 2009). Its widely recognized pathophysiology is based on four factors: follicular hyperkeratinization, colonization of *Propionibacterium acnes* (*P. acnes*), increased sebum production, and inflammation (Webster, 2002). In particular, *P. acnes* contribute to the inflammatory reaction of acne by inducing monocytes to produce pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-8 (Vowels et al., 1995). During the inflammatory reaction, *P. acnes* activate monocyte cytokine release through a toll-like receptor (TLR)-dependent pathway (Jugeau et al., 2005).

TLRs are components of the innate immune system, providing germline encoded receptors that recognize evolutionarily conserved pathogen associated molecular patterns (Netea et al., 2004). TLRs are involved in host defense against a variety of pathogens including bacteria, parasites, and fungi (Kim et al., 2002; Netea et al., 2004). It has been shown to play a critical role in the initiation and resolution of inflammation by inducing both activation and apoptosis of macrophages (Into et al., 2004; Lopez et al., 2003). Also, TLR2 induces NF-κB activation and apoptosis of THP-1 human monocytic cells mediated by MyD88, Fas-associated death domain protein, and caspase-8 (Aliprantis et al., 1999, 2000). Another arm of innate immune response is production of nitric oxide by macrophages. Inducible nitric oxide synthase (iNOS) is regulated by cytokines especially interferon (IFN)-γ, and it generates nitric oxide and other toxic nitrogen radicals that are lethal to a variety of intracellular pathogens (Chan et al., 1995; Jacobs et al., 1996).

Many pathogens have evolved specific mechanisms to avoid, alter, or disable the antimicrobial effects of monocytes/macrophages (Kato et al., 2013). Several pathogenic bacteria participate

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in an essential pathogenic mechanism associated with promotion of inflammation via apoptosis of monocytes/macrophages. Furthermore, bacterial organisms promote the destruction of monocytic phagocytes by apoptosis, thus circumventing the first line of defense of the immune system (Muro et al., 1999; Zychlinsky and Sansonetti, 1997). However, it is not yet known what degree of acne vulgaris is enough to induce pro- or anti-apoptotic mechanisms. As such, the inhibition of THP-1 monocytic cell apoptosis would serve as a reasonable target in the development of a therapeutic strategy for acne vulgaris.

Melittin is a cationic, hemolytic peptide that is the main toxic component in the venom of the honey bee (*Apis mellifera*). It has multiple effects, including anti-bacterial, anti-viral, and anti-inflammatory effects, in various cell types (Raghuraman and Chattopadhyay, 2007). Recent studies have shown that melittin can induce cell cycle arrest, growth inhibition, and apoptosis in various tumor cells (Chu et al., 2007; Zhang et al., 2007). However, the cellular mechanism and the anti-apoptotic effect of melittin in *P. acnes*-induced THP-1 cells have not been explored. In the present study, we investigated the anti-inflammatory and anti-apoptotic mechanism by examining the effect of melittin on *P. acnes*-induced THP-1 monocytic cells.

2. Materials and methods

2.1. Preparation of bacteria

P. acnes (ATCC 6919) was obtained from the Korean Culture Center of Microorganisms (Seoul, Korea) and cultured at 37 °C on reinforced clostridium medium (BD, MD, USA) under anaerobic conditions before assay. *P. acnes* was cultured on tryptic soy broth (BD, MD, USA) with 5% (v/v) defibrinated sheep blood at 37 °C until reaching $OD_{600}=1.0$ (logarithmic growth phase). The log phase bacterial culture was centrifuged at 5000*g* at 4 °C for 15 min, and the culture supernatant was removed, filtered (through a 0.22 μm pore size filter), and used immediately or stored at −20 °C. The bacterial pellet was washed three times in 100 ml of phosphate buffer saline (PBS) and finally suspended in 10 ml of PBS. The *P. acnes* suspension was incubated at 80 °C for 30 min to achieve a heat-killing reaction. The heat-killed *P. acnes* suspension was stored at 4 °C until use.

2.2. Cell cultures

A human monocytic cell line THP-1 cell was obtained from the American Type Culture Collection and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% antibiotics. Cells were cultured in a humidified incubator at 37 °C in a 5% CO_2 atmosphere. THP-1 cells (1.0×10^6 cells/ml) were seeded in complete medium. 24 h later, the cells were changed to fresh serum-free medium containing the indicated concentrations of melittin (0.1, 0.5, and 1 μg/ml; Sigma, Mo, USA). After 30 min, the cells were treated with heat-killed *P. acnes* ($1.0\times10^{5-7}$ CFU/ml, PA), the culture supernatant of *P. acnes* (50 μl/ml, CS), and lipopolysaccharide (LPS, 100 ng/ml) co-cultured for 8 h. Following this, the cells and culture supernatants were collected.

2.3. Cell viability assay

Cell viability of THP-1 cell was determined by the cell counting kit (CCK)-8 assay (Dojindo, Kumamoto, Japan). The THP-1 cells were seeded in a 96-well plate at 1.0×10^4 cells/well and incubated with different concentrations of melittin for 8, 12, and 24 h. After experimental treatment, 10 μl of WST-8 solution [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] was added to each well, and the THP-1 cells were incubated for an additional 4 h at 37 °C. The absorbance values were measured at 450 nm using a microplate reader.

THP-1 cells were washed two times with PBS and treated with a 0.4% solution of trypan blue and visualized as clear cells under a microscope. THP-1 cells that are no longer viable, and have damaged membranes that allow entry of the dye, stain blue. Assays were performed in triplicate and repeated at least three times. The number of intact viable cells was expressed as percentage of total cells.

2.4. LDH assay

To measure the extent of damage to the cells, the stable cytosolic enzyme resulting from cell lysis, lactate dehydrogenase (LDH) was measured in the cell culture medium using an LDH Cytotoxicity Assay Kit (Takara Biomedicals, Otsu, Japan). Simply stated, the clear medium (100 μl/well) was transferred into an optically clear 96-well plate, then 100 μl of LDH Reaction Mix was added to each well, mixed and incubated at room temperature for 30 min. The absorbance at 490 nm was measured by a microplate reader.

2.5. Enzyme-linked immunosorbent assay (ELISA)

THP-1 cells (1.0×10^6 cells/ml) in serum-free medium were stimulated with 1.0×10^7 CFU/ml of heat-killed *P. acnes*, alone or in combination with the indicated concentrations of melittin (0.1, 0.5, and 1 μg/ml) and were incubated for 8 h. The culture supernatants were then harvested. The concentrations of TNF-α and IL-8 in culture supernatant were measured using an ELISA kit (R&D System, MN, USA). The O.D was measured at 450 nm in an ELISA reader (BMG Labtechnologies, Mornington, Germany).

2.6. Preparation of protein fractions and Western blotting

Proteins in cell lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA). The membrane was blocked with 5% skim milk in Tris buffered saline-Tween and incubated with primary antibody at 4 °C overnight. The membrane was incubated with a horseradish peroxidase-conjugated secondary antibody and visualized by enhanced chemiluminescence according to the manufacturer's instructions (Amersham, NJ, USA) and exposed to Kodak X-OMAT film. Primary antibodies used in this study were the following: anti-IKKα, anti-phospho-IKKα, anti-IκBα, anti-phospho-IκBα, anti-NF-κB, anti-phospho-NF-κB p65, anti-caspase-3, anti-caspase-8, and anti-PARP which were purchased from Cell Signaling Technology (MA, USA). Also, anti-TNF-α from Abcam (MA, USA), and anti-TLR2, anti-TLR4, anti-IL-1β, and anti-glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) from Santa Cruz (CA, USA) were used. All primary antibodies were diluted at 1:1000. Signal intensity was quantified by an image analyzer (Las 3000, Fuji, Japan).

2.7. Flow cytometric cell cycle analysis

THP-1 cells were plated at a density of 1.0×10^6 cells/ml in a total volume of 10 ml and exposed for 24 h at 37 °C to 1.0×10^7 CFU/ml of heat-killed *P. acnes* or melittin. After treatment, cells were collected by centrifugation for 5 min at 4000*g*, re-suspended in 300 μl of ice-cold phosphate-buffered saline (PBS), fixed in ice-cold 95% ethanol and stored at −20 °C for at least 30 min. Fixed cells were collected by centrifugation and resuspended in PBS containing RNase A (500 UI/mL). Samples were kept