Expression and Modulation of LL-37 in Normal Human Keratinocytes, HaCaT cells, and Inflammatory Skin Diseases

Defensins and cathelicidins (LL-37) are major antimicrobial peptides (AMPs) of the innate immune system of the human skin. In normal non-inflamed skin these peptides are negligible, but their expression can be markedly increased in inflammatory skin disease such as psoriasis. We designed this study to identify the expressions of LL-37 in normal human keratinocyte (NHK) and HaCaT cells after exposure to stimulants and to investigate difference of LL-37 expression accompanied with cell differentiation status, and come to understand difference of susceptibility to infection in atopic dermatitis and psoriasis. Expressions of LL-37 in NHKs and HaCaT cells were evaluated by using RT-PCR, Western blotting, and immunohistochemical (IHC) staining at 6, 12, and 24 hr post stimulation after exposure to Ultraviolet B irradiation and lipopolysaccharide. And expression of LL-37 in skin biopsy specimens from patients with atopic dermatitis and psoriasis was determined by immunohistochemical analysis. In time-sequential analyses of LL-37 expression revealed that LL-37 was expressed in NHKs, but not in HaCaT cells. IHC analysis confirmed the presence of abundant LL-37 in the epidermis of psoriasis. Therefore we deduced that expression of LL-37 is affected by UV irradiation, bacterial infection, and status of cell differentiation.

Key Words : CAP18 lipopolysaccharide-binding protein; LL-37; Keratinocytes; HaCaT Cell; Psoriasis; Dermatitis, Atopic

INTRODUCTION

Human skin is constantly at risk of damage with exposure to environmental insults including microbial challenge and ultraviolet irradiation (1). So innate immune system may immediately respond to intruding microbes for prevention of further invasion, although stratum corneum is the first line of defense. Antimicrobial peptides (AMPs), which is a kind of innate immune system, can be isolated from various tissues including respiratory, urogenital, and skin epithelium (2, 3). In mammals, several AMPs such as defensins, cathelicidins, cecropins, histatins, lactoferrins, NK-lysin, and protegrins have been found (1, 4, 5). These peptides, which are produced by keratinocytes in the skin, can disrupt the membrane of the target microbe or penetrate the microbial membrane, interfering with intracellular functions (1-5). The mechanisms that regulate expressions of AMPs are not yet understood. Recently, however, Toll-like receptor (TLR) 2 and TLR 4 have been suggested to be related with the expressions of these AMPs (6, 7).

Among numerous antimicrobial peptides, human β-defensin (HBD) and cathelicidin are the major peptides in mammalian skin and they can be induced by injury or inflammation of the skin (1, 8-10). In humans, the cathelicidin family is known to contain just a single member, LL-37 (9, 10).

Precise roles of HBD and LL-37 for the protection of UV-induced damage in the skin are less well understood. Yang et al. (11) reported that HBD may recruit immature dendritic cells and memory T cells through their interaction with chemokine receptor 6 (CCR6) and therefore play important roles in both innate and adaptive immune responses.

Atopic dermatitis and psoriasis, the common inflammatory skin diseases, show contrasting disease features although both diseases are characterized by defective skin barrier (12). About 30 percent of patients with atopic dermatitis have bacterial or viral infections of the skin, as compared with only 7 percent of patients with psoriasis (13). This fact is due to distinct profile of chemokines and incoming helper T cell types, and difference of epidermal thickness (13). In psoriasis, epidermis is thickened owing to increased and accelerated differentiating keratinocytes. So, quantitatively, abundant antimicrobial peptides are expressed in the epidermal layer of psoriasis (12, 14).

Normal human keratinocytes (NHK) which are normally differentiated cell and HaCaT cells which are immortalized, rarely differentiated keratinocyte cell lines show different differentiation feature. So, we designed this study to identify the expressions of LL-37 in NHK and HaCaT cells after exposure to stimulants in order to elucidate their roles for environmental insults and to investigate the level of LL-37 expres-
sion accompanied with cell differentiation status and come to understand difference of susceptibility to infection in atopic dermatitis and psoriasis.

**MATERIALS AND METHODS**

**HaCaT cell culture**

The HaCaT cells, human keratinocyte cell lines, were cultured in IsoCove's Modified Dulbecco's Medium (IMDM) (Gibco, Carlsbad, CA, U.S.A.), supplemented with 10% fetal bovine serum (Gibco) and penicillin-streptomycin 100 IU/100 μg/mL (Gibco) grown on 75 cm² flask and incubated at 37°C for 6, 12, and 24 hr. Cultured HaCaT cells were divided as a number of 2 × 10⁵/mL and plated in a standard flat bottomed 10 cm² polystyrene plate. Cells were starved in IMDM supplemented with free fetal bovine serum overnight, some cells then were irradiated Ultraviolet B (UVB) 20 mJ/cm² and treated lipopolysaccharide (LPS) 2.5 g/mL, 5.0 g/mL, respectively and incubated for 6, 12, and 24 hr.

**Normal human keratinocytes**

For harvesting NHKs, neonatal foreskin was obtained from neonatal circumcision specimen and then primary culture was done. Briefly, neonatal foreskin was chopped in 1 mm size and trypsinized in room temperature overnight. After vortex vigorously and incubated for 5 min and supernatant was taken and plated on 25 cm² culture flask and then incubated 5% CO₂ at 37°C in keratinocyte growth media (Clonetics, East Rutherford, NJ, U.S.A.). Cultured normal human keratinocytes were irradiated in UVB 20 mJ/cm² and treated LPS 2.5 μg/mL, 5.0 μg/mL (Sigma, St. Louis, MO, U.S.A.) respectively and incubated for 6, 12, and 24 hr.

**Ultraviolet B irradiation**

Dosage of irradiation was 20 mJ/cm² which were chosen based on preliminary data in this experiment. UVB irradiation was delivered with a Philips TL 20W/12 (Eindhoven, Netherlands), a fluorescent bulb emitting 280-320 nm wave with a peak at 313 nm wave. Before UVB irradiation, medium was removed and covered with phosphate buffered saline (PBS). Irradiation output was monitored by means of a Waldmann UV-meter (Waldmann, Villigen-Schwenningen, Germany).

**Lipopolysaccharide stimulation**

2.5 μg/mL and 5.0 μg/mL of LPS (Sigma, St. Louis, MO, U.S.A.) was used.

**Preparation of primer**

We synthesized the PCR primer from the basis of GenBank data. Primers were chemically synthesized by using DNA synthesizer (Pharmacia, B) rkgetan, Uppsala, Sweden). Their sequences were as follows:

LL-37 (348 bp) :  
5′ -TCG GAT AAC CTC TAC CG-3′ (sense),  
5′ -GGG TAC AAG ATT CCG CAA AA-3′ (anti-sense)

GAPDH (593 bp) :  
5′ -CCA CCC ATG GCA AAT TCC ATG GCA-3′ (sense),  
5′ -GGT GCT GCT TGT TAG GAG GTC AAG TAA AGG GC-3′ (anti-sense)

**Reverse transcription-polymerase chain reaction**

Total RNA was isolated from NHKs and HaCaT cells using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.), cells were added 1 mL of TRizol reagent in cultured dish. After 5 min at room temperature, added 0.2 mL of chloroform per 1 mL of TRizol reagent, shook tubes vigorously by hands for 15 sec and incubated them at 15°C to 30°C for 5 min. The mixtures were centrifuged with 12,000 rpm at 4°C for 15 min, transferred the upper aqueous phase to a fresh tube, and the same amount of 2-propanol was added. After mixtures were incubated at 4°C for 15 min, it was centrifuged with 12,000 rpm at 4°C for 15 min. The supernatant was removed, then washed 500 μL of 70% ethanol with 12,000 rpm at 4°C for 5 min, the RNA pellet was briefly dried. The purified RNA was dissolved in DEPC-DW 30 μL. 3 μg of total cellular RNA was reverse transcribed at 42°C for 30 min in a 20 μL volume containing 1 μL reverse transcriptase (TaKaRa, Shiga, Japan), 10 × buffer 2 μL, 10 mM dNTP 2 μL (dNTP mix), oligo dT primer 1 μL, RNase inhibitor 0.5 μL, 25 mM MgCl₂ 4 μL, 2 μL of each cDNA sample from the RT-PCR was amplified by PCR in 25 μL containing 10 μL buffer 2.5 μL, 25 mM MgCl₂ 2.5 μL and 10 pmol 0.75 μL primer. Reactions were cycled 35 times with denaturation at 94°C for 1 min followed by annealing at 59°C for 1 min and finally an extension step at 72°C for 1 min.

**Electrophoresis**

The products were run on 1.5% agarose gel containing 1 μg ethidium bromide per millimeter. 20 μL of reaction mixture was mixed with loading buffer separated by electrophoresis for 15 min at 100 voltages and visualized by UV transillumination.

**Quantitative analysis**

It has been quantitatively analyzed with densitometer that...
hybrids of PCR products of LL-37 and GAPDH on DIG chemiluminescent film were calculated (volume of LL-37/volume of GAPDH × 100).

**Western blotting**

NHK and HaCaT cell were lysed in a buffer containing 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 µg/mL phenylmethanesulfonyl fluoride (PMSF), 1 µg/mL aprotinin, 1% Triton X-100, and centrifuged with 12,000 rpm at 4°C for 30 min.

The supernatant was transferred into new tube, 30 µg of soluble protein were loaded in 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with sample buffer containing 1 M Tris, glycerol 50%, samples were heated at 95°C for 5 min prior to gel loading.

For LL-37 detection, separated protein on gel electrophoresis was transferred to nitrocellulose membrane (Osmonics, Minnesota, MN, U.S.A.) at 0.16A for 1 hr. The membrane was washed 3 times with Tris-buffered saline Tween 20 (TBST), and blocked with 5% skim milk for 1 hr at room temperature. Following this, the membrane were incubated overnight at 4°C with goat anti-human LL-37 polyclonal antibody (1:1,500 in blocking solution, SantaCruz, Delaware, CA, U.S.A.) and then washed 3 times with TBST.

The secondary mouse anti-goat peroxidase conjugated antibody (1:1,500 in 5% bovine serum albumin, SantaCruz, California, CA, U.S.A.) was incubated for 1 hr at room temperature.

After washing the membrane with TBST, the membrane was developed with ECL solution (SantaCruz) for 3 min then exposed to radiography film (Roche, Indianapolis, IN, U.S.A.).

**Immunohistochemistry (IHC) for LL-37**

The HaCaT cells and NHKs were cultured on coverslip (Nunc, Rochester, NY, U.S.A.); the cells were fixed for 10 min in 4% paraformaldehyde and washed 3 times for 5 min with PBS. Endogenous peroxidase was inactivated by incubating 3% hydrogen peroxide at room temperature for 5 min, and blocked with 3% BSA for 20 min then washing. Cells were incubated overnight at 4°C with goat anti-LL-37 polyclonal antibody, the primary antibody was diluted 1:50 in PBS, rinsed three times with PBS and incubated with mouse anti goat peroxidase-conjugated antibody (1:200 in PBS) for 1 hr at room temperature. After washing 3 times, cells were immersed in 3,3′-diaminobenzidine (DAKO, Glostrup, Denmark), rinsed in distilled water.

Also immunohistochemical staining was carried out using sections of paraffin-embedded tissues of psoriasis and atopic dermatitis. Briefly, sections 4 µm thick were deparaffinized in xylene three times for 5 min each time, and epitopes were retrieved by autoclaving (121°C) for 10 min in citrate-buffered saline (pH 6.0). After cooling for 20 min at room temperature, endogenous peroxidase activities were quenched with 3% H2O2 treatment for 5 min. Sections were blocked with normal goat serum for 1 hr and incubated with goat anti-human LL-35 polyclonal antibody (1:200 dilution in PBS). After five washes with PBS, sections were incubated with peroxidase-conjugated anti-goat secondary antibody, and color was developed with diaminobenzidine.

**Statistical analysis**

The amount of LL-37 expression in NHK and HaCaT cell between unstimulated control and stimulated groups were statistically compared using t-test.

**RESULTS**

**RT-PCR**

Expression of LL-37 mRNA in HaCaT cells was not detected in unstimulated control. Moreover, in UVB irradiation and LPS stimulated groups, expressions of LL-37 mRNA were not observed in HaCaT cells (data not shown).

However, those in NHKs were upregulated by UVB irradiation and LPS stimulation (Fig. 1). At 6, 12 hr post stimulation, LL-37 mRNA expressions were more markedly upregulated by LPS 2.5 µg/mL and at 24 hr, by UVB 20 µJ/cm2. These results were statistically significant compared to control group (p<0.001).

**Western blotting**

Expression of LL-37 protein in NHKs was evaluated by Western blotting study using polyclonal antibody to LL-37 at 6, 12, and 24 hr after stimulation (Fig. 2). The expression amounts of LL-37 protein in UVB irradiated and LPS treated groups were shown more intense than those of un-
stimulated control group. Expression of LL-37 band were more intense in 6 hr, 12 hr after stimulation than 24 hr after stimulation. But expressions of LL-37 protein in HaCaT cells were not detected by Western blotting study (data not shown).

Immunohistochemistry

The expressions of LL-37 protein in NHKs were determined by IHC analysis. In NHKs, expressions of LL-37 protein of UVB irradiated and LPS stimulated groups were more strongly stained than those of unstimulated groups (Fig. 3). Expressions of LL-37 protein in HaCaT cells were not detected by IHC study using polyclonal antibody to LL-37 at 6, 12, and 24 hr after stimulation (data not shown). Also IHC confirmed the presence of abundant LL-37 in the epidermis of psoriasis. The sample from psoriatic lesions had much more intense staining for LL-37 than sample from atopic dermatitis lesions (Fig. 4).

DISCUSSION

Naturally occurring AMPs are critical component of innate immune system which provide mammalian skin protection against invasive bacterial infection and other environmental insults including UV irradiation, toxin, extreme temperature etc. (1, 2). In human, the two main antimicrobial peptide families are defensins and cathelicidins. The two defensin subfamilies, α- and β-defensins, differ in the length of peptide segments between the six cysteines and the pairing of the cysteines that are connected by disulphide bonds (8). The α-defensins are abundant in neutrophil granules and Paneth cells. The β defensins have been classified to β defensin 1, 2, 3, and 4 which can be expressed constitutively or after exposure to stimulants (3). Until now many studies have been reported regarding defensins and those of cathelicidins are less reported in comparison with defensins (4, 5). So, we intended to elucidate that inducible expression of LL-37 after exposure to different experimental conditions and their role in skin immunity against various environmental insults including bacterial infection and UV irradiation in this study.

The term ‘cathelicidin’ was introduced in 1995 to encompass bipartite molecules containing both cathelin domain and C-terminal antimicrobial peptide domain (6). Cathelicidins are widely expressed family of mammalian antimicrobial peptides and like many such molecules are synthesized as a preproprotein. This preproprotein consists of highly conserved signal sequence and cathelin domain but have substantial heterogeneity between species in the C-terminal domain.
Expression and Modulation of LL-37

encoding the mature active peptide. Porcine and bovine neutrophils contain a variety of cathelicidins, whereas there is the only one cathelicidin in human known as human cationic antimicrobial peptide 18 kDa (hCAP-18). The major effector molecule of hCAP-18 is C-terminus which begins with 2 leucines and 37 amino acid residues in length, so called LL-37 (8). The antimicrobial activity of cathelicidins is only activated following proteolytic processing from the cathelin domain of the preproprotein by elastase or proteinase-3 (15-17).

LL-37 has broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria such as *S. aureus*, *Pseudomonas aeruginosa*, and *E. coli*, as well as against fungi and enveloped viruses and expression of it is up-regulated by inflammatory stimulants or injury such as microbial invasion (18-20). Because LL-37 is widely distributed within skin and mucosal epithelial tissues, as well as secretion such as saliva and sweat, it is ideally situated to serve a sentinel role as multifunctional effectors of innate immunity (4, 5, 16). Beside this function, LL-37 can influence wound repair and has been shown to have additional effects on the host. LL-37 up-regulates epithelial expressions of chemokine and chemokine receptor genes, and itself acts as chemoattractant for neutrophils, monocytes, T cells and mast cells (21, 22).

We could observe that LL-37 expressions were up-regulated by LPS stimulation and UVB irradiation in NHKs. In RT-PCR analysis, at 6 hr and 12 hr post stimulation, LL-37 is more intensely expressed in LPS 2.5 μg/mL stimulated group but, at 24 hr post stimulation. LL-37 expression was also markedly up-regulated by UVB irradiation with dose of 20 mJ/cm². However, expressions of LL-37 were not seen in HaCaT cells.

Production patterns of LL-37 proteins were also identified in Western blotting in NHKs. The maximum expressions of LL-37 protein in NHKs were obtained with LPS stimulation at 6 hr post stimulation. But, LL-37 expressions were not enhanced even in stimulated HaCaT cells. In immunohistochemical staining analysis, UVB irradiated and LPS treated groups were stained stronger than those of control. However, in HaCaT cells, the study showed no staining.

We obtained the results that expressions of LL-37 were up-regulated in NHKs by UVB irradiation and LPS stimulation. No expressions of LL-37 in HaCaT cells even exposure to UVB irradiation and LPS could be supposed on the assumption of the following hypotheses. First, these results may be due to different feature of differentiation between these two groups. That is NHKs are gradually differentiating, whereas HaCaT cells are really not differentiating any more. Such dissimilar differentiation property may have exerted influence on the expression of LL-37. In the second place, we could presume that there were genetic defect encoding LL-37 or unknown interference on transcription of LL-37 in HaCaT cells. The third, we assumed that relative deficiency of immunity in perinatal period brought about these results. Dorschner et al. (23) reported that human cathelicidin expression is significantly elevated in the perinatal period when compared with adult skin and expression is present in the absence of inflammation. In consideration of relative deficiency in cellular immune function in neonates, it is attractive to speculate that augmented innate antimicrobial peptide defense mechanism is beneficial to the newborn and provides essential level of microbial protection. In this study NHKs were prepared from neonatal foreskin, so LL-37 is more prominently expressed in NHKs independent of stimulation in comparison with HaCaT cells.

Atopic dermatitis is very common skin disease known to be associated with a high prevalence of skin infections, however, in psoriasis only small proportion of patients are suffering from infection. In atopic dermatitis, inflammation is mediated by type 2 helper T cells which can produce IL-4, IL-13 whereas inflammation of psoriasis is mediated by type 1 helper T cells and epidermis is thickened owing to increased differentiated keratinocytes (12-14). And LL-37 has potential binding sites for acute phase response factor (APRF) and IL-6, in its promoter and intron regions (24). Interestingly, increased levels of IL-6 and IL-8 have been identified in psoriatic skin.

Therefore, bacterial infection is not severe problem in psoriasis although the barrier function of psoriatic skin is disrupted because of increased expression of AMPs (12). However these peptides are significantly decreased in acute and chronic lesions of atopic dermatitis. Decreased expressions of AMPs may account for the susceptibility of atopic dermatitis patients to skin infection and inhibitory effects of IL-4 and IL-13 on HBD-2 expression may account for this finding (13, 14, 25).

The results of immunostaining for LL-37 in the sample of psoriatic lesions and atopic dermatitis lesions were consistent with previous study showing that psoriatic lesions have greater expression of LL-37 than atopic dermatitis. In a word, significant difference of LL-37 expression between psoriasis and atopic dermatitis may be due to distinct cytokine profiles and difference of epidermal differentiation status.

With our experiments, we were able to demonstrate enhanced expression of LL-37 after LPS stimulation and UVB irradiation in both mRNA and protein level and confirmed that LL-37 expression is induced by inflammatory stimulations. And LL-37 expressions were also affected by cell differentiation status. LL-37 expressions were identified only in NHKs which are gradually differentiated cells and more abundant in the epidermis of psoriasis. In summary, we could understand susceptibility difference to infection in atopic dermatitis and psoriasis that show different epidermal differentiation status and concluded that induced LL-37 may contribute to the skin immunity against various environmental insults.

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