Regulation of Human Beta-Defensin 3(hBD-3) in Human Keratinocyte(HaCaT) Cell Lines

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Background : **The large surfaces of the skin are often initial site of contact between microorganism and human. The skin are coated with epidermis and epithelial cells can recognize microorganism and mount a fast defense through the production of various inducible antibiotic peptides. This leads to chracteristic broad spectrum of antimicrobial activity against bacteria, fungi, and viruses. Recent studies introduce us new peptides with antimicrobial activity such as β,-defensins and cathelicidins. They are expressed on the epithelia and polymorphonuclear leukocytes, which are first lines of defence from various invasive environments. Futhermore, they are considered very interesting and important endogenous antibiotics. Our previous study has shown that the expression of human defensin(hBD-2) mRNA, which is potent antibiotic peptide against Gram-negative bacteria(*P. aeruginosa*), was upregulated with ultraviolet(UV) irradiation, tumor necrosis factor-α(TNF-α) and lipopolysaccharide(LPS) in HaCaT cells. A novel hBD-3, 5-kDa, nonhemolytic antimicrobial peptide, was demonstrated a salt-insensitive broad spectrum of potent antimicrobial activity against many potentially pathogenic microbes in especially, multiresistant S. aureus. We have analyzed the expression patterns of hBD-3 in HaCaT cell lines.**

Objective : **This research have done in order to evaluate the expression and regulation of hBD-3 mRNA in human keratinocyte cell lines.**

Methods : **HaCaT cell lines were used to all culture experiments. Cultured human keratinocytes were stimulated with UV irradiation or TNF-α or LPS to determine whether hBD-3 mRNA production occurred. Reverse transcription-polymerase chain reaction (RT-PCR) was performed to amplify hBD-3 cDNA from stimulated keratinocytes in a time dependant manner, and densitometry was used to verify the specificity of RT-PCR amplication products.**

Results : **Expression of hBD-3 was upregulated with UV irradiation, TNF-α and LPS in HaCaT cells compared to control**

Conclusions : **Human keratinocytes are capable to induce hBD-3 mRNA, as well as hBD-2, in response to UV irradiation, TNF-α and LPS. suggesting that these cells could play an important role against the bacterial infection and UV light damage in human skin.**

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Key Words : Human β, defensin, Tumor necrosis factor-α, Lipopolysaccharide, Ultraviolet irradiation

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INTRODUCTION

Defensin is a gene family with antimicrobial peptides and thought to be effector molecules in innate immunity. Defensins are cationic, cystein-rich antimicrobial peptide components of the mammalian innate system[1]. They are members of a supergene family consisting of α and β, subtypes, located in a cluster at chromosome 8[2]. These two

classes of defensin differ in their disulfide bond pairing, genomic organization, and in their tissue distributions. In humans, the α-defensins, namely human neutrophil defensins 1-4 and human defensin 5-6 in Paneth cells and other epithelia, are known[3,4]. Two β-defensins of epithelial origin, hBD-1 and hBD-2, have been identified and characterized in humans. hBD-1 is constitutively expressed in epithelial cells of the urinary and respiratory tract and oral cavity[5]. Whereas hBD-2 was isolated from psoriatic skin and is expressed in skin and epithelia of the respiratory and gastrointestinal tract and gingival epithelium in response to pro-inflammatory stimuli[6,7,8]. Our previous study has shown that the expression of human defensin(hBD-2) mRNA, which is potent antibiotic peptide against Gram-negative bacteria(*P. aeruginosa*), was upregulated with ultraviolet(UV) irradiation, tumor necrosis factor-α(TNF-α) and lipopolysaccharide(LPS) in HaCaT cells[9].

Both human β-defensins 1 and 2 show microbicidal activity predominantly against Gram-negative bacteria like *E. coli*, *P. aeruginosa*, yeasts and virus[10,11,12,13]. In contrast to hBD-1 and hBD-2, hBD-3 is more potent antimicrobial peptide and expressed a low baseline level not only in epithelial but also in nonepithelial tissues such as tonsils, airway and gingival keratinocytes, heart, and skeletal muscle[14,15]. Despite of many information of expression of hBD-3 at various tissue site, little is known about the factors that regulate the expression of this peptide, especially under the condition that immunological reaction occurred.

Recent investigations showed that hBD-3 mRNA also was induced by bacteria and TNF-α [13]. But other investigations have demonstrated that hBD-3 mRNA expression was not upregulated after application of *P. aeruginosa* or cytokine such as TNF-α but upregulated significantly in response to IFN-γ[14]. Thus we focused on TNF-α, UV irradiation, and LPS and this research was done in order to evaluate the expression and regulation of hBD-3 mRNA in human keratinocyte cell lines.

MATERIALS AND MATHODS

1. Cell culture

We used normal human keratinocytes, HaCaT cell line for all culture experiments. For harvesting normal human keratinocyte, neonatal foreskin was obtained from circumcision specimen. These cell lines were kindly given by Prof. Hwang (Soonchonhyang University, Seoul, South Korea).

Cells were grown to 90% confluence in Isocoves modified Dulbecco's medium (IMDM) media supplemented with 10% fetal bovine serum (FBS).

2. Ultraviolet B irradiation

Doses of irradiation (50mJ/cm^2) were chosen based on previous literature on irradiation in culture[16,17]. UV 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 UV irradiation, medium was removed, and covered with phosphate buffered saline (PBS). Irradiation output was monitored by means of a Waldmann UV-meter (Waldmann, Villigen-Schwenigen, Germany).

3. Tumor necrosis factor-α stimulation

Cultured keratinocytes were stimulated by 100 U/ml human recombinant tumor necrosis factor-α (TNF-α) (Boehringer Mannhein, Germany).

4. Lipopolysaccharide stimulation

Two hundreds ng per ml of *Escherichia coli* 026:B6 lipopolysaccharide (LPS) (Sigma Chemical Co., St. Louis, MO) was used.

5. Preparation of primers

We synthesized the PCR primers from the basis of Gene Bank data. Primers were chemically synthesized by using DNA synthesizer (Pharmacia, Lid.). The sense primer sequences of hBD-3 was 5'-AGC CTA GCA GCT ATG AGG ATC-3' and antisense was 5'-CTT CGG CAG CAT TTT GCG CCA-3' with the expected product size of 206 base pairs. The sequence of the sense primer of GAPDH was 5'-CCACCCATGGCAAATTCCATGGCA-3' and the anti-sense primer was 5'-GGTGC-TGCTTGTTAGGAGGTCAAGTAAAGGGC-3' with an expected product size of 593 bp.

6. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured human keratinocytes, which were incubated with medium alone or UV irradiation (50 mJ/cm^2) or TNF-α

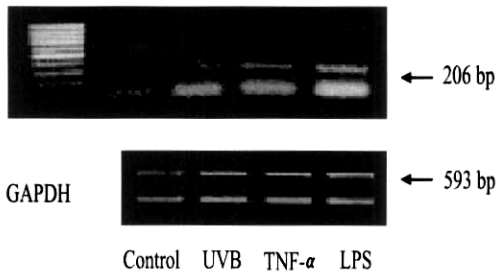

Fig 1. Expression of hBD-3 mRNA in cultures of normal human keratinocytes from neonatal foreskin at 12 h (control, UVB 50 mJ/cm^2, TNF-α (100 U/ml), LPS (200 ng/ml)).
In our study with human keratinocytes obtained from neonatal foreskin, expressions of hBD-3 mRNA were not observed in control group. After addition of UVB irradiation with the dose of 50 mJ/cm^2, TNF-α or LPS, expressions of hBD-3 mRNA were upregulated.

(100 U/ml) or LPS (200 ng/ml) at specific time intervals (3, 6, 12, 24 and 48 h) with RNA STAT-60 reagent (Tel-test, USA).

We synthesized first-strand cDNA from 3μg of each total cellular RNA using a first strand cDNA synthesis kit (#K1612 MBI Fermentas, Vilnius, Lithuania). Three micrograms of RNA, 1 μl of Oligo (dT) 18 primer (0.5 g/ml), and DEPC-DW (Diethyl pyrocarbonate, Sigma Chemical Co., USA-distilled water: RNase free buffer) were mixed up until it reached 28μl, which was incubated at 70℃ for 7 min and then added with 10 μl of 5 x reaction buffer (250 mM Tris Hcl (pH 8.3), 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT), 2.5μl of ribonuclease inhibitor (20 U/μl) and 5 ß° of 10 mM dNTP mix (incubate at 37℃). 4.5μl solution of M-MuLV reverse transcriptase (20 U/μl) was added (5 min, total volume of solution 50μl) and then incubated at 37℃ for 1 h.

Synthesized cDNA was incubated at 70℃ for 10 min and stored at -70℃

PCR amplification was performed with a 5μl of cDNA sample from each cell culture in a total volume of 50μl including 1μl of each primer set, 5 ß° of 10 x reaction buffer (250 mM Tris Hcl (PH 8.8 at 25℃), 500 mM Kcl, 0.8 Nonidet P40, MBI, Fermentas Vilnius, Lithuania), 0.5μl of Taq. polymerase (5 U/μl), 2μl of 2.5 mM dNTP mix and 35.5μl of DEPC-DW in eppendorft tubes.

The amplification of reversibly transcribed (RT) and synthesized cDNA was carried out using specific primer pairs. PCR amplification was performed with a Gene Amp PCR System 9600 (Perkin Elmer, USA). The PCR conditions for hBD-3 were (i) 95℃ for 5 min (initial denaturation), (ii) 95 ℃ for 15 sec (denaturation), (iii) 60℃ for 5 sec (annealing), (iv) 72℃ for 10 sec (extension), and (v) 72℃ for 5 min (final extension).

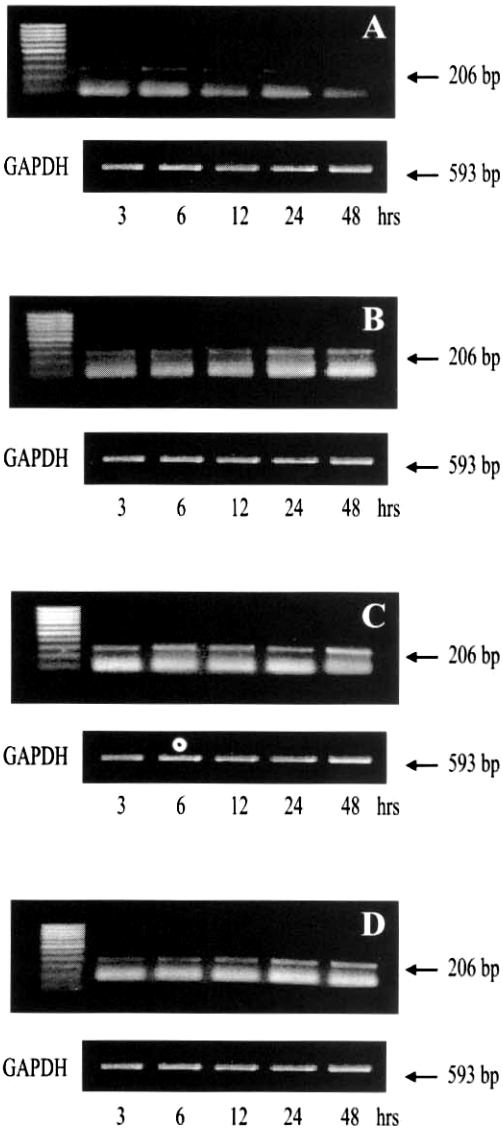

Fig 2. (A) Expression of hBD-3 mRNA in cultures of HaCaT cells with medium alone, (B) UVB 50 mJ/cm^2, (C) TNF-α (100 U/ml), (D) LPS (200 ng/ml). In unstimulated HaCaT cells, hBD-3 mRNA were weakly detected at any of time points treated. After addition of UVB irradiation or TNF-α or LPS, hBD-3 mRNA expression was upregulated at 6, 24 and 48 h post stimulation.

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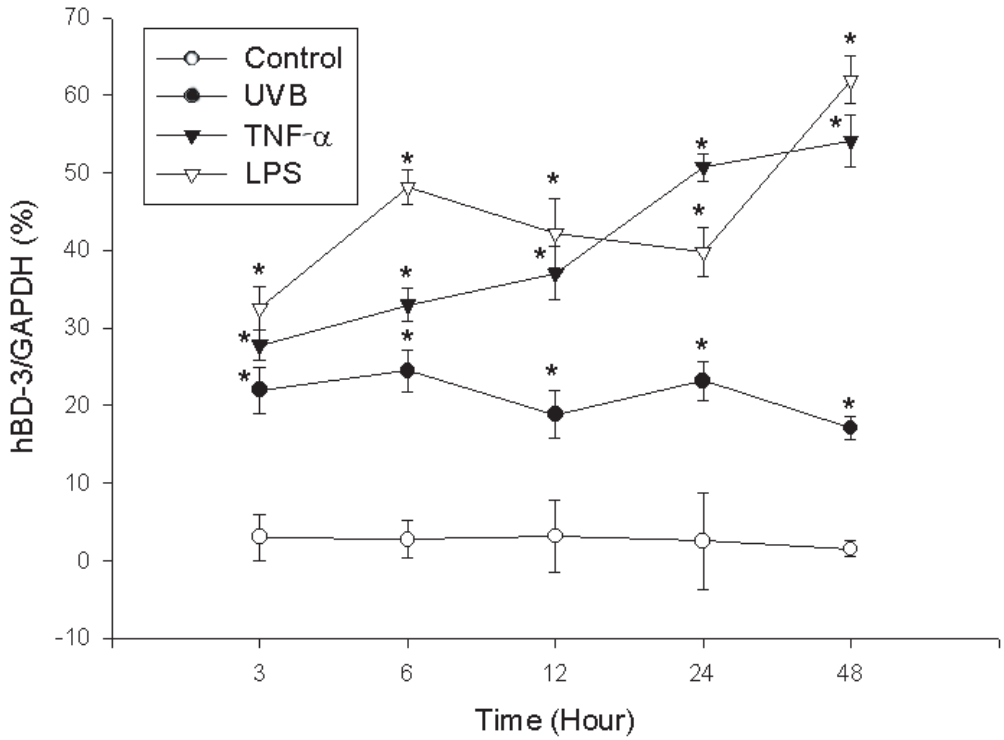

Fig 3. Expression of hBD-3 in HaCaT cell line. Expression of hBD-3 mRNA was upregulated with UVB irradiation, TNF-α and LPS in HaCaT cells and in comparison to the control, significantly higher at 6-12 h post stimulation with LPS and peak at 24-48 h post stimulation with UVB 50 mJ/cm^2 and TNF-α (*p<0.05).

The step (ii) to (iv) were repeated 35 cycles for hBD-3. The constitutively expressed glyceraldehyde phosphate dehydrogenase (GAPDH) gene served as the internal control to ensure that equal amounts of RNA were added to each sample.

The amplified PCR products were visualized after electrophoresis in 2% agarose gels (Sigma Chemical Co., USA) containing ethidium bromide (0.5μg /ml) (Sigma Chemical Co., USA).

7. Quantitative analysis

It has been quantitatively analyzed with densitometer that hybrids of PCR products of hBD-3 and GAPDH on DIG chemiluminescent film were calculated. (volume of β-defensins/volume of GAPDH $\times$100).

8. Statistical analysis

For the statistical comparison of the amounts of human β-defensin 3 for each human keratinocytes between unstimulated control and stimulated groups, the Student's t-test was used(n=3). The means and standard devations were calculated and P-values <0.05 were considered to be significant.

RESULTS

1. Expression of hBD-3 mRNA in cultures of human keratinocytes from neonatal foreskin.

In our study with human keratinocytes obtained from neonatal foreskin, expressions of hBD-3 mRNA were not detected in control group. After addition of UV irradiation with the dose of 50 mJ/cm^2, TNF-α or LPS, expressions of hBD-3 mRNA were upregulated(Fig. 1).

2. Expression of hBD-3 mRNA in cultures of HaCaT cells was upregulated by UVB irradiation or TNF-α or LPS stimulation.

In unstimulated HaCaT cells, hBD-3 mRNA were weakly detected at any of time points treated. After addition of UVB irradiation with the dose of 50 mJ/cm or TNF-α, hBD-3 mRNA expression was upregulated and peaked at 24 to 48 h post

stimulation(Fig 2, 3). With LPS, hBD-3 mRNA expression was also upregulated and peaked at 6 to 12 h post stimulation (Fig 2, 3). The expression level of hBD-3 mRNA at post stimulation in the treated groups with the the dose of 50 mJ/cm^2 or TNF-α or LPS was significantly higher than those of untreated groups($p<0.05$)(Fig 3).

DISCUSSION

Defensins are a family of peptides thought to provide an innate immune defense at epithelial interface with external microbial pathogens[10,18]. They are also believed to promote a rapid cellular immune response to infection via chemotactic effect on monocyte[19]. In addition to their antimicrobial actions, defensins may accelerate wound healing by virtue of their mitogenic effect on epithelial cells and fibroblasts20. And other investigations revealed that hAD also have the ability to attract T cells and human hBD attract immature dendritic cells and memory T cells via the chemokine receptor CCR6, providing a link between innate epithelial defense and adaptive immunity[21].

Defensins are members of a supergene family consisting of α and β subtypes. In human, the α-defensins (hAD) has six isoforms, which are expressed by polymorphonuclear leukocytes(hAD 1-4) and Paneth cells(hAD 5, 6)[3,10]. The β defensins(hBD) are expressed at epithelium. The first isolated human β-defensin, hBD-1, was purified from hemofiltrates and was later found in urine as a Gram-negative bacteria-killing antibiotics[22]. The mRNA of this antimicrobial peptide is constitutively expressed in epithelial cells of the urinary and respiratory tract and oral cavity[5]. The second form, hBD-2, was discovered in extracts of psoriatic scales[23]. hBD-2 is expressed in inflamed skin, respiratory and gastrointestinal tract and gingival epithelium[7,11,13,23,24] and induced expressed in response to inflammatory stimuli such as IL-1, TNF-α UV light, and contact with bacteria, virus and fungus[9,13,25,26]. Very recent paper proposed that the expression of hBD-2 peptide by human keratinocytes required differentiation of the cells as well as a cytokine or bacterial stimulus[27].

HBD-1 and hBD-2 have a broad spectrum of antimicrobial activity. But, they demonstrate only low microbicidal activity against Gram-positive bacteria such as *S. aureus*. These Gram-positive bacterial infection is a major cause for skin infections, in particular in atopic dermatitis, but no problem of healthy human skin. The growing infections caused by multiresistant Gram-positive bacteria demand to detect systemic endogenous *S. aureus*-killing factors, which is novel antimicrobial peptide hBD-3.

HBD-3 exhibits the characteristic properties of the β-defensin family. hBD-3 is a 5-kDa, nonhemolytic antimicrobial peptide and is isolated from human psoriatic scales. HBD-3 mRNA is expressed not only in epithelial but also in nonepithelial tissues such as heart, skeletal muscle, esophagus, gingival keratinocyte and trachea[14,15]. In addition, it is detected in the placental membranes suggesting that the peptide may play a role in fetal-maternal defenses during pregnancy[28]. hBD-3 gene is located 13 kb upstream from the HBD-2 gene, supporting the idea that all human β-defensins are clustered on chromosome 8[28]. The cDNA sequence is ~43% identical to HBD-2 and shares the β-defensin six cysteine motif[28]. However, antimicrobial spectrum, the regulation, expression pattern, and other functions of hBD-3 differ significantly from other β-defensins. HBD-3 demonstrated a salt-insensitive broad spectrum of potent antimicrobial activity against many cutaneous pathogenic microbes including multiresistant *S. aureus*[15].

In our study, hBD-3 mRNA expression was detected in normal HaCaT cells under normal culture conditions. The level of hBD-3 mRNA, when compared with normal control, was increased under the stimulation of TNF-α, UVB and LPS. TNF-α induces cytokeratin synthesis through a transcriptional complex containing NF κ B(nuclear transcriptional factor kappa B), indicating that keratinocyte cytokines are capable of regulating cytoskeletal proteins that become activated during injury and inflammation. Bacterial subcapsular components such as lipopolysaccharide (LPS) are potent inducers of inflammation that results after infection. UV light also may be an important environmental factor that modulates keratinocyte functions in many ways via NF κ B pathway. From our previous study, we examined the expression pattern of hBD-1 and hBD-2 under the same stimulation in the same culture media and explained the relationship between the defensins and binding sites for NF κ B[9].

These results indicate that HaCaT cells may be a source of local hBD-3 synthesis and hBD-3, like

hBD-2, can be induced by proinflammatory cytokines such as TNF-α, UVB and bacterial components in epithelial cells at physiologically condition. These data provide the first evidence that hBD-3 may contribute to protect the skin from UV damage. hBD-3 represents also human β-defensin family where expression is regulated by inflammatory stimuli at a transcriptional level.

In conclusion, hBD-3 is a defensin expressed in epithelial and non-epithelial tissues in contrast to earlier statements that β-defensins are characteristically expressed in epithelial tissues. It may also play a role to enhance skin protection against microorganism invasion and to defend human from environmental insult.

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