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질상피세포에서 Toll-like Receptor Ligands에 의한 항생 펩티드 Human β-defensin-3의 발현조절

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Modulation of Antimicrobial Peptide Human β-defensin-3 by Toll-like Receptor Ligands in Vaginal Epithelial Cells

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Purpose: Vaginal epithelial cells have always been exposed to various pathogens. However, this has not always caused clinical infection. In addition to a previously reported protection effect of the vagina, currently, the innate immune response is thought to be important as one of the causes explaining the phenomenon. Therefore, we investigated the innate immunity of the vagina and related mechanisms in infected vaginal epithelial cells focusing on the antimicrobial peptide human β-defensin-3 (HBD-3).

Materials and Methods: We investigated the signaling molecules, Toll-like receptors (TLRs), through which mammals sense infection in vaginal epithelial cells, with activation with lipopolysaccharide (LPS), *Staphylococcus aureus* peptidoglycan (PGN), or zymosan. Reverse transcriptase-polymerase chain reaction analysis of HBD-3 messenger RNA expression in vaginal epithelial cells after treatment with three pathogens was performed for investigation of pathogen-associated molecular patterns. Then, we also studied the following mechanism of innate immunity of the vagina focusing on HBD-3 in vaginal epithelial cells infected with gram-positive bacteria, gram-negative bacteria, or fungus.

Results: Vaginal epithelial cells (VK2/E6E7 cells) constitutively expressed TLR2 and TLR4 and produced antimicrobial peptide HBD-3 upon activation with LPS, PGN, or zymosan. VK2/E6/E7 cells exposed to LPS, PGN, or zymosan showed increased p38 mitogen activated protein kinase (MAPK) activity. In addition, LPS-, PGN-, and zymosan-induced HBD-3 expression was attenuated by SB203580, a p38 MAPK inhibitor, emphasizing the importance of p38 MAPK in induction of HBD-3.

Conclusions: Vaginal epithelial cells may contribute to the host innate immune defense upon exposure to gram-negative bacteria, gram-positive bacteria, or fungi in the vagina by upregulation of HBD-3 expression.

Keywords: Vagina; Beta defensin 3, human; Toll-like receptors

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INTRODUCTION

Humans have always been exposed to various pathogens, including bacteria, funguses, and viruses. However, they have not always caused clinical infections. These phenomena were thought to be related to the innate immune response. In women, the vaginal mucosa, which is composed of squamous epithelial cells, is a target site of vaginitis and can be a site of microbial pathogen colonization, which causes genitourinary tract infection. The most common pathogens are yeasts (e.g., *Candida* species), gram-positive bacteria, and gram-negative bacteria. However, the vaginal mucosa has a powerful innate immune system that protects the female reproductive organs from infection. Several defense mechanisms control vaginal immunity. Previously, the physical barrier effect of the stratified squamous epithelium and the acidic environment due to the lactic acid produced by indigenous microflora, lactobacilli, were thought to be important.[1] Recently, the interest in vaginal innate immunity has been moved to the immune mechanism of epithelial cells activated by pathogens, receptors, and mediators and the product, antimicrobial peptides.

Toll-like receptors (TLRs) are the principal signaling molecules through which mammals sense infection. Eleven different mammalian TLRs have been identified to date, and their ligands include molecular products derived from bacteria, fungi, viruses, and protozoa.[2,3] TLR2 recognizes cell wall components such as peptidoglycan (PGN), lipoteichoic acid, and lipoprotein from gram-positive bacteria and zymosan from the yeast cell wall. Lipopolysaccharide (LPS) is the major component of the outer cell membrane of gram-negative bacteria, and it is recognized by TLR4.[4] Each TLR has different coreceptors or adaptors to respond to activation. TLR4 interacts with cluster of differentiation 14 (CD14) and myeloid differentiation protein 2 (MD-2). The signaling mechanism of TLR2 and TLR4 is mediated by a cytoplasmic adaptor protein, myeloid differentiation factor 88 (MyD88).[5] Once ligands are recognized by TLRs by being transmitted by two major signaling pathways, mitogen activated protein kinase (MAPK) and nuclear factor-κB (NF-κB), acute responses including the expression of pro-inflammatory cytokines, chemokines, and antimicrobial peptides occur.[2,6,7] These locally produced antimicrobial peptides, chemotactic factors, and proinflammatory cytokines mediate the sequence of events leading to the elimination of pathogens, including the recruitment of leukocytes to the sites of infection and the activation of the adaptive immune response.[2,8]

Antimicrobial peptides play an important role in the innate immunity of mammalian species.[9] Human β-defensins (HBDs) (HDB-1, 2, and 3) are antimicrobial peptides that are produced in the epithelial cells of various organs and phagocytic cells. Beta-defensins are cysteine-rich peptides of 36-42 amino acids in length and are stabilized by three disulfide bonds.[10] Among the three human β-defensins (HBD-1, HBD-2, and HBD-3), HBD-1 is produced in the gastrointestinal tract, respiratory tract, mammary gland, skin, pancreas, and kidney. HBD-2 is expressed in the gastrointestinal tract, respiratory tract, and pancreas, and HBD-3 is strongly expressed in skin, trachea, tongue, and tonsils.[11,12] HBD-2 and HBD-3 expression is primarily constitutive, and it is inducible by cytokines such as tumor necrosis factor alpha and interleukin-1, various microorganisms, LPS, and other microbial products.[13-15] Unlike other HBDs, HBD-3 is a salt-insensitive defensin with broad antimicrobial activity against multidrug-resistant nosocomial strains.[12,16,17]

Concerning vaginal innate immunity, little is known about the mechanism by which epithelial cells are activated by the pathogens, receptors, and mediators involved in the immune response. A previous study reported HBD-2 expression and NF-κB activation in vaginal epithelial cells.[2] In the present study, we focused on HBD-3 and the MAPK pathway in vaginal epithelial cells infected with gram-positive bacteria, gram-negative bacteria, or fungus.

MATERIALS AND METHODS

The expression of TLR messenger RNA (mRNA) adaptor protein MyD88, which is used by all TLRs except TLR3 to activate the NF-κB, and cluster of differentiation 14 (CD14) and MD-2, which are coreceptors for LPS, was assessed by reverse transcriptase-polymerase chain reaction (RT-PCR) in VK2/E6E7 cells, an immortalized normal human vaginal cell line. THP-1 cells, a human monocyte cell line that expresses all known TLRs except TLR6, were used as the positive control. Phosphate buffered saline (PBS) was used as the negative control.

To assess the biological relevance of the expression of multiple TLRs in vaginal epithelial cells, we investigated the effects of pathogen-associated molecular patterns representing gram-negative bacteria, gram-positive bacteria, and fungi on the expression of antimicrobial peptides HBD-2 and HBD-3.

Cell surface receptor-mediated signaling activates MAPK in various cells. We examined the role of MAPK in the induction of HBD-3 in VK2/E6E7 cells by inhibiting MAPK. To evaluate if LPS-, PGN-, and zymosan-induced HBD-3 expression in VK2/E6E7 cells is mediated by p38 MAPK, we introduced 20 μM SB203580, a specific inhibitor of p38 MAPK, along with LPS, PGN, or zymosan in VK2/E6E7 cells.

1. Cells and Stimulations

The immortalized human vaginal epithelial cell line (VK2/E6E7 cells) was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in Keratinocyte-SFM (Gibco BRL, Eggstein, Germany) supplemented with 5 ng/ml recombinant epidermal growth factor, 50 μg/ml bovine pituitary extract, antibiotic/antimycotic solution (Sigma-Aldrich, St. Louis, MO, USA), and 20 mM L-glutamine (Sigma-Aldrich) in a humidified atmosphere containing 5% CO_2. The medium was changed every 2 days.

LPS (purified from *Escherichia coli* 026:B6), PGN, and zymosan (from *Saccharomyces cerevisiae*) were purchased from Sigma. VK2/E6E7 cells were treated with either LPS (1 μg/ml), PGN (5 μg/ml), zymosan (10 μg/ml), or a microbial compound-free control medium for 0, 3, 6, 12, or 24 hours. After the indicated times, cells and supernatants were collected for further analysis.

2. RNA Isolation and RT-PCR

Total cellular RNA was extracted from cells using an RNeasy kit (Qiagen, Seoul, Korea). Total RNA (2 μg) was reverse transcribed to cDNA by using a Moloney Murine Leukemia Virus Reverse Transcriptase kit (Promega, Madison, WI, USA). After reverse transcription (RT), the cDNA was amplified using PCR with gene-specific primers designed to amplify a portion of the coding sequences (Table 1). The PCR consisted of one cycle of 10 min denaturation at 94°C, 18-35 cycles of 1 min at 94°C, 1 min at 48-55°C, and 1 min at 72°C, with a final extension step at 72°C for 10 min. Beta-actin was amplified to confirm that an equal amount of total cDNA was used for each sample, and all experiments were conducted in duplicate. Template cDNA prepared from THP-1, a normal human monocyte cell line that expresses all known TLRs except TLR6, was used as a positive control for detecting TLR mRNA in VK2/E6E7 cells. Sequencing was performed on all positive PCR products (Macrogen, Seoul, Korea) to confirm the identity of the amplified genes.

3. Immunoblotting

Total protein extracts from VK2/E6E7 cells were prepared in a lysis buffer of 1.5% sodium dodecyl sulfate (SDS), 62.5 mM Tris-HCl (pH 6.8), 5 mM ethylenediaminetetraacetic acid, 1% 2-mercaptoethanol, 1 μg/ml antipain dihydrochloride, 1 μg/ml chymostatin, and 1 μg/ml leupeptin (all from Sigma-Aldrich, Seoul, Korea). Lysates were cleared by centrifugation, and supernatants were stored at -80°C. The constituent proteins of the epithelial cell lysates were separated by SDS/PAGE on a 10% separating gel and then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). The gel and the nitrocellulose membrane were stained using Coomassie Brilliant Blue and Ponceau S (all from Sigma), respectively, to verify equivalent loading of protein in the wells. Membranes were blocked by incubation in Tris-buffered saline (150 mM NaCl, 25 mM Tris [pH 7.4]), containing 0.05% Tween 20 (Sigma) and 3% nonfat dry milk (Sigma), for 2 hours at room temperature (RT) and subsequently incubated overnight at 4oC with a 1:1,000 dilution of the primary antibodies (rabbit anti-phospho-p38 MAPK and rabbit anti-β-actin) (Cell Signaling Technology Inc., Danvers, MA, USA). Horseradish peroxidase conjugated goat anti-rabbit IgG (Cell Signaling Technology) was used

Table 1. Primer sets used in polymerase chain reaction analysis

Gene	Primer sequence	Product size
HBD-2		255
Sense	TGCCTCTTCCAGGTGTTTTT	
Antisense	ATGGCTTTTTGCAGCATTTT	
HBD-3		206
Sense	AGCCTAGCAGCTATGAGGATC	
Antisense	CTTCGGCAGCATTTTCGGCCA	
β-actin		375
Sense	CTCGGTGAGGATCTTCATGA	
Antisense	CCATCGAGCACGGCATCGTC	

HBD: human β-defensin.

as the secondary antibody, diluted 1:10,000 in blocking buffer, and membranes were incubated for 1 hour at RT. Blots were developed using enhanced chemiluminescence (GE Health-Care, Piscataway, NJ, USA) as the substrate.

4. Immunocytochemistry

VK2/E6E7 cells were grown on glass coverslips, fixed with 4% paraformaldehyde for 40 min, and then permeabilized with 0.1% Triton X-100 for 20 min at RT. Cells were treated with 3% bovine serum albumin (Sigma-Aldrich) for 2 hours to block nonspecific binding of the antibodies and incubated with antibodies against HBD-3 (6 μg/ml, AF4435; R&D Systems, Minneapolis, MN, USA) at 4°C overnight. After washing, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG (1:400 dilution, F7367; Sigma-Aldrich) for 1 hour at RT, and DNA was counterstained with propidium iodide (Sigma-Aldrich). Coverslips were mounted with Fluorescence Mounting Medium (DAKO, Carpentaria, CA, USA). Fluorescence was detected by confocal laser microscopy (Zeiss LSM 510, Oberkochen, Germany).

RESULTS

VK2/E6E7, a normal human vaginal epithelial cell line, was screened for mRNA expression of all known human TLRs and the accessory molecules MD-2, CD14, and MyD88 by RT-PCR. The expression of TLR2 and TLR4 mRNA was detected in VK2/E6E7 cells. The expression of accessory molecules such as MyD88, CD14, and MD-2 was also detected (Fig. 1).

When semiquantitative RT-PCR analysis of HBD-3 mRNA expression in immortalized vaginal epithelial cells after treatment with LPS, *Staphylococcus aureus* PGN, or zymosan was performed, LPS, PGN, and zymosan had an extremely strong stimulatory effect on HBD-3 mRNA expression in VK2/E6E7 cells at 3, 6, 12, and 24 hours after stimulation (Fig. 2).

Pathogen-associated molecular patterns induced p38 MAPK activation in VK2/E6E7 cells. Inhibiting p38 MAPK resulted in reduced HBD-3 mRNA expression and protein production (Fig. 3).

LPS, *Staphylococcus aureus* PGN, and zymosan induced p38 MAPK phosphorylation in VK2/E6E7 cells. The activation of p38 MAPK by detecting the phosphor-p38 MAPK protein was observed (Fig. 4).

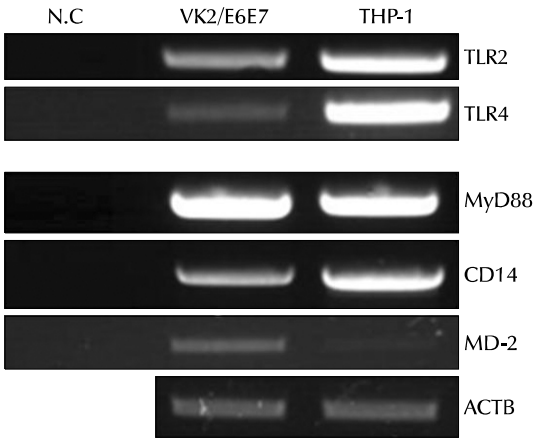

Fig. 1. Expression of Toll-like receptors (TLRs) and accessory molecules in cultured human vaginal epithelial cells. VK2/E6E7, a normal human vaginal epithelial cell line, was screened for mRNA expression of all known human TLRs and the accessory molecules myeloid differentiation protein 2 (MD-2), CD14, and myeloid differentiation primary response gene 88 (MyD88) by reverse transcriptase-polymerase chain reaction. The expression of TLR2 and TLR4 mRNA was detected in VK2/E6E7 cells. The expression of accessory molecules such as MyD88, CD14, and MD-2 was also detected. N.C: normal control, ACTB: β-actin.

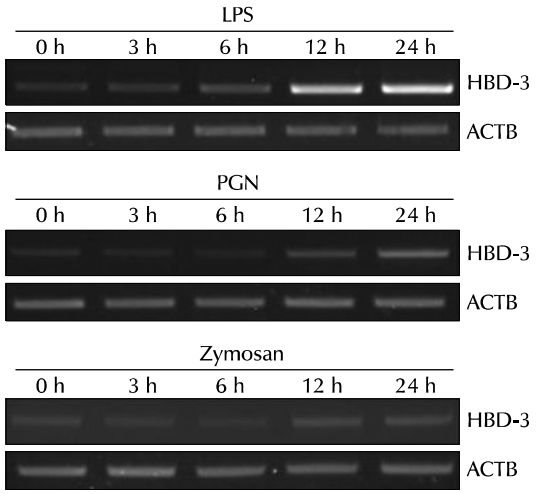

Fig. 2. Pathogen-associated molecular patterns, representing gram-negative and gram-positive bacteria as well as fungal pathogens, induced human β-defensin-3 (HBD-3) in vaginal epithelial cells. Semi-quantitative reverse transcriptase-polymerase chain reaction analysis of HBD-3 mRNA expression in immortalized vaginal epithelial cells after treatment with lipopolysaccharide (LPS), *Staphylococcus aureus* peptidoglycan (PGN), or zymosan was performed. LPS, PGN, and zymosan had an extremely strong stimulatory effect on HBD-3 mRNA expression in VK2/E6E7 cells at 3, 6, 12, and 24 hours after stimulation. ACTB: β-actin.

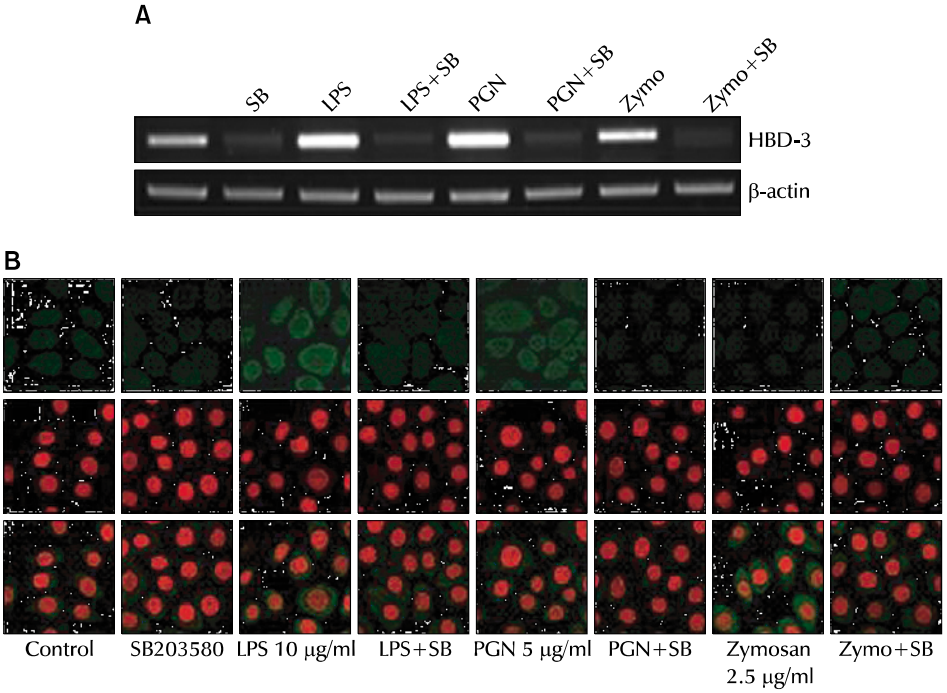

Fig. 3. The specific inhibitor of p38 MAPK (SB203580) attenuates lipopolysaccharide (LPS)-, *Staphylococcus aureus* peptidoglycan (PGN)-, and zymosan-induced human beta-defensin-3 (HBD-3) expression in VK2/E6E7 cells in the mRNA level (A) and the protein level (B).

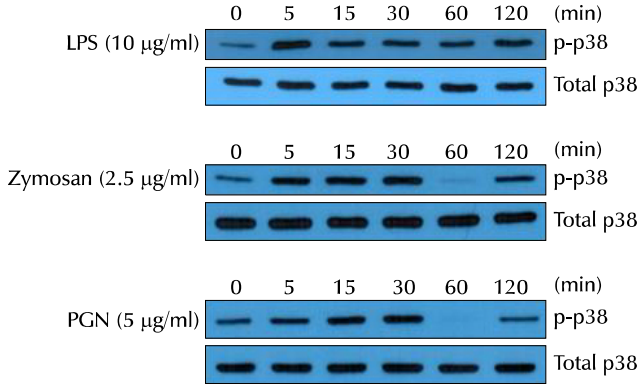

Fig. 4. Lipopolysaccharide (LPS), *Staphylococcus aureus* peptidoglycan (PGN), and zymosan induced p38 mitogen-activated protein kinase (MAPK) phosphorylation in VK2/E6E7 cells. The activation of p38 MAPK by detecting the phosphor-p38 MAPK protein was observed.

DISCUSSION

The tissues of the female reproductive tract are exposed to a large number of infectious agents; therefore, vaginal infections represent an extremely common medical problem.[18] Bacterial vaginosis represents 60% of all vaginal infections and is the major cause of gynecologic morbidity, infertility, ectopic pregnancy, and preterm labor.[18] The stratified squamous epithelium of the vagina represents a physical barrier to pathogenic microorganisms, which is further supported by an indigenous microflora and an acidic environment that is antagonistic against a variety of bacteria related to vaginal tract infections.[19] While the physical barrier function of vaginal epithelial cells was formerly believed to play the major role in protection against infections, observations of anti-*Candida* activity mediated by vaginal epithelial cells indicate that vaginal epithelia play an important, active role in innate immunity.[20,21] As epithelial cells of mucosal surfaces are the first contact with pathogens, we investigated antimicrobial peptide production in the VK2/E6E7 vaginal epithelial cell line in response to microbial compounds from gram-negative and gram-positive bacteria as well as fungi.

There are 11 members of the human TLR family. Pivarcsi et al.[2] noted that TLR2 and TLR4 were expressed at both the mRNA and protein levels in the human vaginal epithelium in vivo and in the human vaginal epithelial cell line PK E6/E7 in vitro.[22] Similarly, we also found that TLR2 and TLR4 were expressed when we treated vaginal epithelial cells with LPS, PGN, and zymosan in this study.

In our previous study, we investigated the expression of HBD-1 and HBD-2 in vaginal epithelial cells treated with LPS and the effects on HBD-2 expressions by 17β-estradiol and progesterone. HBD-1 was produces constitutively in vaginal epithelial cells and the production of HBD-1 was not influenced by LPS, 17β-estradiol and progesterone, but the production of HBD-2 was increased inducibly by LPS.

17β-estradiol and progesterone did not change the production of HBD-2 in normal state, but 17β-estradiol increased the production of HBD-2 and progesterone suppressed the production of HBD-2 under the circumstances with infection.[23] However, it was previously unknown whether HBD-3 is expressed in the vaginal epithelial cells and increases with vaginitis. In this study, VK2/E6E7 cells produced the antimicrobial peptide HBD-3 upon activation with LPS, PGN, or zymosan. This finding suggests that pathogen-associated molecular patterns induce HBD-3 in vaginal epithelial cells. HBD-3 expression is controlled by a tight regulatory network involving NF-κB and activator protein-1.[24-26] These transcription factors are activated by complex signaling pathways, including MAPK.[27] The regulatory mechanisms of HBD-3 production in vaginal epithelial cells were also previously unknown. In this study, LPS-, PGN-, and zymosan-induced HBD-3 expression was attenuated by SB203580, a p38 MAPK inhibitor, emphasizing the importance of p38 MAPK in the induction of HBD-3. These results indicate that VK2/E6E7 cells may contribute to the host innate immune defense upon exposure to gram-negative bacteria, gram-positive bacteria, or fungi in the vagina by upregulating HBD-3 expression.

With these results, we were able to find the new antimicrobial peptide in the vaginal epithelial cells and reveal the mechanism of its increased expression in these cells.

There are some limitations to this study. First, this study was performed for the vaginal epithelial cell line in vitro. Further study is necessary in vivo. Second, this study was performed with only one vaginal epithelial cell line. Further study with other vaginal epithelial cell lines would be required to verify these results. Because the HBD-2 and NF-κB pathway have been studied previously,[2] this study focused on the HBD-3 and MAPK pathway. Therefore, other antimicrobial peptides and pathways were not examined. Because the immune mechanism is very complex, further investigation into these antimicrobial peptides and pathways is necessary. HBD-3 is just one factor contributing to the immune mechanism.

Although further studies are necessary, we expect to use HBD-3 as a diagnostic tool and to promote the innate immunity of patients with recurrent vaginitis in the future.

CONCLUSIONS

Vaginal epithelial cells (VK2/E6E7 cells) expressed TLR2 and TLR4 and produced the antimicrobial peptide HBD-3 upon activation with LPS, PGN, or zymosan. Induction of HBD-3 expression in the vaginal epithelial cells after exposure to LPS, PGN, and zymosan was attenuated by p38 MAPK inhibitor, emphasizing the importance of p38 MAPK in the induction of HBD-3. Therefore, vaginal epithelial cells may contribute to the host innate immune defense upon exposure to gram-negative bacteria, gram-positive bacteria, or fungi in the vagina by upregulating HBD-3 expression.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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