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Original article

Microbial compounds induce the expression of pro-inflammatory cytokines, chemokines and human β-defensin-2 in vaginal epithelial cells

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Abstract

Vaginal epithelium has a powerful innate immune system that protects the female reproductive organs from bacterial and fungal infections. In the present study, we aimed to explore whether the Toll-like receptor (TLR) signaling pathway and the induction of pro-inflammatory cytokines and antimicrobial peptides could contribute to the protection against pathogenic microorganisms in vaginal epithelia, using an immortalized vaginal epithelial cell line PK E6/E7 as a model. We found that TLR2 and TLR4 receptors are expressed in vivo in the vaginal epithelia and in vitro in PK E6/E7 vaginal epithelial cell line. The Gram-negative cell wall compound lipopolysaccharide (LPS), the Gram-positive compound peptidoglycan (PGN), heat-killed *Candida albicans* and zymosan significantly ($P < 0.05$) induced the expression of pro-inflammatory cytokines and chemokines such as TNF-α and IL-8/*CXCL8* in vaginal epithelial cells. Furthermore, the expression and production of human β-defensin-2 (hBD2), an antimicrobial peptide with chemotactic functions, was also up-regulated in PK E6/E7 cells after treatment with LPS, PGN or *C. albicans*. Treatment of vaginal epithelial cells with microbial compounds induced the activation and nuclear translocation of NF-κB transcription factor, a key element of innate and adaptive immune responses. In our work, we provide evidence that microbial compounds induce the production of pro-inflammatory cytokines, chemokines and antimicrobial peptides in vaginal epithelial cells. In vivo, vaginal epithelial cell-derived inflammatory mediators and antimicrobial peptides may play important roles in vaginal immune responses and in the elimination of pathogens from the female reproductive tract.

Keywords: Toll-like receptors (TLRs); Innate immunity; Antimicrobial peptides; Fungal infections; Female reproductive tract

1. Introduction

The innate immune system represents an ancient system of host defense with striking structural and functional similarities among various organisms such as insects and humans. Innate immune recognition is based on a set of germline-encoded pattern recognition receptors (PRRs), which recognize conserved pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) are the principal signaling molecules through which mammals sense infection [1,2]. To date, 11 different mammalian TLRs have been identified, and their ligands include molecular products derived from bacteria, fungi, viruses and protozoa [1,3].

Recognition of ligands by TLRs leads to a series of signaling events including the activation of NF-κB transcription factor resulting in induction of acute responses, the expression of pro-inflammatory cytokines, chemokines and antimicrobial peptides [4–7]. Locally produced antimicrobial peptides, chemotactic factors and pro-inflammatory cytokines are presumed to play a pivotal role in the host defense of epithelial surfaces [8]. They 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 adaptive immune responses [9].

Abbreviations: hBD2, human β-defensin-2; IL-1α, interleukin-1 alpha; IL-8, interleukin-8; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa B; PAMPs, pathogen-associated molecular patterns; PGN, peptidoglycan; PK E6/E7, immortalized vaginal epithelial cell line; TNF-α, tumor necrosis factor alpha.

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Vaginal epithelium has a powerful innate immune system that protects the female reproductive organs from infections. 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 showing antagonistic activity against a variety of bacteria related to vaginal tract infections [10]. While the physical barrier function of vaginal epithelial cells was formerly believed to play the major role in the protection against infections, observation of anti-*Candida* activity mediated by vaginal epithelial cells indicated that vaginal epithelia plays an important, active role in innate immunity [11,12]. However, still little is known about the mechanism of epithelial cell activation by pathogens and the receptors and mediators involved in the immune response.

Here we report that TLR2 and TLR4 are expressed at both mRNA and protein levels in the human vaginal epithelium in vivo and in the immortalized human vaginal epithelial cell line PK E6/E7 in vitro. We show that microbial compounds representing Gram-negative, Gram-positive and fungal pathogens induce the activation of NF-κB transcription factor and the expression of pro-inflammatory cytokines and chemokines, which may play a crucial role in the mobilization of leukocytes and the initiation of adaptive immune responses at the sites of infection. Furthermore, we provide evidence that microbial compounds induce the expression of human β-defensin-2 (hBD2), an antimicrobial peptide highly effective against a broad range of pathogens, in vaginal epithelial cells.

2. Materials and methods

2.1. Cells and stimulations

The immortalized human vaginal epithelial cell line (PK E6/E7 cells) was a kind gift of Professor Schaeffer (Northwestern University Medical School, Chicago, IL, USA) [13]. PK E6/E7 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, MA, USA) and 20 mM L-glutamine (Sigma) in a humidified atmosphere containing 5% CO_2. The medium was changed every 2 days.

Lipopolysaccharide (LPS, purified from *Escherichia coli* (026:B6)), *Staphylococcus aureus* peptidoglycan (PGN, Product Nr.: 77140) and zymosan (*Saccharomyces cerevisiae*) were purchased from Sigma. *Candida albicans* (0656 CBS Delft) was cultured on Sabourand agar and transferred to fresh agar 24 h prior to the specified experiments. *Candida* cell suspension was prepared in Keratinocyte-SFM, the cells were killed by incubating them for 30 min at 56 °C. *Mycobacterium tuberculosis* cell wall extract was purchased from Human Ltd. (Gödöllő, Hungary). The extract contains the 19-kDa antigen of *M. tuberculosis*.

PK E6/E7 cells were treated with either LPS (1 μg/ml), PGN (5 μg/ml), heat-killed *C. albicans* (10 *Candida*/PK E6/E7 cell), zymosan (10 μg/ml), tuberculin (5 μg/ml) or with microbial compound-free control medium for 0, 3, 6, 12 or 24 h. After the indicated times, cells and supernatants were collected for further analysis.

2.2. Quantitative reverse transcription polymerase chain reaction (Q-RT-PCR)

Quantitative real-time PCR analyses were performed as previously described [14] (Nagy et al., in press). Briefly, total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First strand cDNA was synthesized from 1 μg of total RNA in a 20 μl final volume using the ImProm II cDNA Synthesis Kit (Promega Corporation, Madison, WI, USA). After reverse transcription, real-time quantitative PCR was used to amplify cDNAs using primers and TaqMan probes specific for human 18S, IL-8, IL-1α, and hBD2 genes, as described earlier [14] (Nagy et al., in press). The primer set used for TNF-α detection was: TNF-α sense: TCTCCTTCCTGATCGTGGC; TNF-α anti-sense: GGTTCAGCCACTGGAGCT. Reaction conditions for real-time PCRs were as follows: 95 °C for 5 min followed by 40 cycles at 95 °C for 15 s (denaturation), and at 57 °C for 45 s (annealing and elongation). Gene-specific PCR products were measured by means of an iCycler IQ real-time PCR continuously during 40 cycles. Alternatively, traditional RT-PCR was performed for qualitative analyses using specific primers sets for TLR2, TLR4 and MyD88, as described previously [14]. To avoid false-positive results due to amplification of contaminating genomic DNA in the cDNA preparation, we used primers spanning exon–exon junctions. Calibration curves were constructed and linearity was achieved for all primer sets at least in three orders of magnitude under the conditions we used in our experiments. The identity of PCR products amplified by the specific primer sets were confirmed by direct sequencing. All PCR assays were performed in triplicates. Target gene expression was normalized between different samples based on the values of the expression of the internal positive control (18S rRNA).

2.3. Immunohistochemistry

Vaginal tissue specimens were obtained from women undergoing colporaphy ($n = 5$). The Ethical Committee of the University of Szeged approved this investigation. Cryostat sections (5 μm) fixed for 15 min in cold methanol (–10 °C) were incubated overnight at 4 °C in a humid chamber with the primary antibodies. For immunohistochemical staining, goat polyclonal antibodies raised against human TLR2 (clone sc-8689) and TLR4 (clone-8694) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:100 dilution were used. Primary antibodies preincubated for 2 h at room temperature with a fivefold excess (by weight) of the specific peptides that have been used to generate the primary antibodies were