Immunology Letters 119 (2008) 84–90

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/

Innate immune responses of epididymal epithelial cells to *Staphylococcus aureus* infection

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ARTICLE INFO

Article history:
Received 6 March 2008
Received in revised form 1 May 2008
Accepted 10 May 2008
Available online 4 June 2008

Keywords:
Epididymal epithelial cells
Staphylococcus aureus
Toll-like receptors
Innate immune responses
p38 MAPK
NF-κB

ABSTRACT

The epithelium is an active participant in the host response to infection. We hypothesized that epididymal epithelia play a role in the innate immune responses by sensing the presence of pathogens, expressing and secreting inflammatory cytokines that recruit inflammatory cells in response to invading pathogens. Our results indicated that TNF-α and IL-1β could be secreted by the primary cultured rat epididymal cauda epithelia infected with *Staphylococcus aureus*. Epididymal epithelial-induced nitric oxide synthase (iNOS) expression was up-regulated after *S. aureus* infection and nitric oxide (NO) was also found to be produced significantly. NF-κB inhibitor BAY11-7082 inhibited TNF-α secretion completely and p38 mitogen-activated protein kinases (MAPKs) inhibitor SB203580 decreased TNF-α secretion partly, indicating that NF-κB and p38 signal pathways were involved in this inflammation response. Toll-like receptor (TLR)-2 and -4 were shown to be expressed in primary cultured rat epididymal epithelia. After infection the level of TLR2 expression was up-regulated rather than TLR4. These results demonstrated that epididymal epithelium have an innate immune response through activation of p38 MAPK and NF-κB after TLR2 activation by *S. aureus* infection.

1. Introduction

The epididymis is the site for the transport, maturation and storage of spermatozoa [1,2]. The epididymis consists of a highly convoluted tubule that links the testis to the vas deferens, the lumen of which is lined by epithelial cells and surrounded by a smooth muscle cell layer.

The epithelial cells play pivotal roles in epididymal function. Firstly, the epithelial cells are essential for epididymal spontaneous contractions involved in spermatozoa transport [3]. Secondly, the functions of epithelial cells, including absorption, secretion, synthesis and metabolism, create an appropriate luminal environment for the acquisition of fertilizing potential and progressive motility of spermatozoa [4,5]. The third important function of the epithelial cells is to inactivate and remove pathogens and thereby prevent infection of the epididymis from pathogens invasion [6].

The epididymal epithelial cells, like other epithelial cells, express and secrete antimicrobial peptides and proteins, such as defensin, mucin, eppin, cationic antimicrobial protein 18, lipopolysaccharide binding protein, sperm antigen 11 [7–25]. These defense effectors protect spermatozoa from invading pathogens through antimicrobial activation. They play key roles in the host defense against invading pathogens.

It is well known that epithelial cells play a bridge role in innate and adaptive immunity. Recognition of pathogens is attributed largely to Toll-like receptors (TLRs) and NODs of epithelial cells which function in innate immunity via recognition of pathogen-associated molecular patterns [26–29]. In response to infection, the epithelial cells recognize pathogens and send initial signals, such as the release of proinflammatory cytokines and chemokines, for recruiting the neutrophils and mononuclear lymphocytes. Epididymis tract expresses multiple TLRs, which demonstrates that TLRs play important roles in innate immunity of epididymis tract [30]. While little is known about TLRs signaling in epididymis. It is unclear what patterns of TLRs expression are in epididymal epithelial cells, furthermore, the molecular mechanisms that epididymal epithelial cells defense responses to pathogen and its roles in innate immune system and adaptive immune system are poorly understood.

The epididymal epithelial cells constitute the first line of defense against pathogens and, therefore, must possess the ability to sense the presence of pathogens and activate adaptive immunity.

Here with an *in vitro* infection model we demonstrated that epididymal epithelial cells serve as a sensor for *Staphylococcus aureus* by secreting multiple cytokines. NF-κB and the p38 signal pathways are responsible for *S. aureus*-induced inflammatory cytokines

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doi:10.1016/j.imlet.2008.05.002

in epididymal epithelial cells. Epididymal epithelial cells express TLR2 and TLR4. TLR2, rather than, TLR4 is up-regulated after *S. aureus* infection for 24 h.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (8–10 weeks old) were purchased from the Animal Center of Sun Yat-sen University. Animals lived in a constant-temperature room (20 °C) with a 12L:12D photoperiod and were allowed food and water *ad libitum*. Animal experiments were performed in accordance with the guidelines of the Sun Yat-sen University Animal Use Committee

2.2. Materials

Eagle minimum essential medium (EMEM), fetal bovine serum (FBS), nonessential amino acids, penicillin/streptomycin, Hanks balanced salt solution, sodium pyruvate, and trypsin were purchased from Gibco Laboratories (NewYork). 5*a*-dihydrotestosterone (5*a*-DHT), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and collagenase I were bought from Sigma Chemical Co. (St. Louis, MO). Bay 11-7082, SP60025, SB203580 and PD98059 were purchased from Calbiochem (Germany). TNF-α and IL-1β ELISA kits were got from ebioscience (USA). Anti-P-p38 and anti-rabbit IG-G were obtained from cell signal company, anti-p-IκB antibody, anti-TLR2 antibody, anti-TLR4 antibody, anti-iNOS antibody and anti-GAPDH antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The bicinchoninic acid (BCA) protein assay kit was bought from Shenenergy, Inc. (Shanghai, China). Trizol was purchased from Invitrogen (USA). M-MLV reverse transcriptase was obtained from Promega (USA). *Taq* polymerase was fromTaKaRa (Dalian, China).

2.3. Epididymis epithelial cells isolation and primary culture

Epididymis epithelial cells were isolated from male Sprague–Dawley rats according to a well-established protocol [31]. In brief, male Sprague–Dawley rats were killed by CO_2 inhalation. Cauda epididymis were dissected out, finely minced with scissors, and treated successively with 0.25% (w/v) trypsin and 0.1% (w/v) collagenase. The disaggregated cells were suspended in EMEM containing nonessential amino acid (0.1 mM), sodium pyruvate (1 mM), 5*a*-DHT (1 nM), 10% FBS, penicillin (100 IU/ml), and streptomycin (100 IU/ml) after 4–6 h primary culture, then seeded onto 6-well culture plate, 12-well culture plate or 96-well culture plate. Cultures were incubated for 4 days at 32 °C in a humidified atmosphere of 5% CO_2. The medium was changed every 2 days.

2.4. Staphylococcus aureus culture and infection procedure

S. aureus (ATCC 6538) used in this study was a gift from Zhi Li doctor of Guang-Zhou medical college. *S. aureus* were inoculated from single colony into LB broth and incubated at 37 °C with shaking. *S. aureus* were centrifuged (10,000 × *g*, 5 min) from LB cultures, washed with PBS three times and resuspended in PBS. The optical density (OD) was determined for *S. aureus* number estimation (A_{600}: 0.8 OD unit = ∼3.3×10^8 cfu/ml). *S. aureus* were added into EMEM without penicillin and streptomycin to get a final concentration 1×10^7 cfu/ml. The epithelial monolayers (90–100% confluency) were washed with PBS and replaced with EMEM containing 1×10^7 cfu/ml *S. aureus*. At different time points after infection, the medium was pipetted out, centrifuged (10,000 × *g*, 5 min) and stored at −80 °C. The epithelial monolayers were washed with PBS and incubated with EMEM containing penicillin and streptomycin.

2.5. Determination of cell viability

Epididymal epithelial cells were seeded into 96-well culture plates. After confluence epididymal epithelial cells were infected with *S. aureus*. After infected at different time points, cells were washed with PBS for three times and volume of 200 μl of 0.5 mg/ml MTT in DMEM medium was added to each well and incubated for 4 h. The medium was removed and 100 μl of dimethyl sulfoxide (DMSO) was added to each well. The formazan dye crystals were solubilized for 15 min, and absorbance at 570 nm was measured using a microplate reader (Labsystems, Finland). The results were expressed as the percentage of MTT reduction, assuming that the absorbance of control cells was 100%.

2.6. ELISA assay for TNF-α and IL-1β release

After the treatment described above, the supernatant was collected to determine TNF-α and IL-1β released using commercially available ELISA kits. Briefly, 96-well plates were coated with capture antibody (anti-TNF-α or IL-1β) overnight, washed with 0.05% Tween-20 in PBS and coated with 10% FBS in order to block non-specific binding. Known concentrations of TNF-α and IL-1β(standard) and supernatant containing the TNF-α and IL-1β released by the cells after treatment at different times were added as aliquots into appropriate wells, incubated for 2 h and decanted from the wells. Anti-TNF-α and IL-1β plus enzyme reagent (biotinylated detection antibody conjugated to Streptavidin–Horseradish) were added and incubated for 1 h. After washing the plate, a solution containing a substrate for the enzyme (TMB-peroxide chromogen) present in the anti-TNF-α or IL-1β + enzyme reagent mixture was added and the plate was incubated for 30 min. The reaction was stopped using a 1 M H_2SO_4 solution and the absorbance was read at 450 nm using a microplate reader (Labsystems, Finland). The absorbances were then used to calculate the TNF-α or IL-1β concentration from the standard curve and adjusted by their dilution factor.

2.7. Measurement of nitrite

NO production in cell culture supernatants was evaluated by measuring the nitrite, a stable end product of NO. Nitrite in the culture supernatants was determined by the Griess reaction [32]. After indicated time periods of incubation, cell-free supernatant of 100 μl was incubated with 100 μl Griess reagent. Samples were incubated at 25 °C for 10 min and absorbance was measured at 540 nm. Concentrations were determined versus a sodium nitrite standard.

2.8. RNA isolation and RT-PCR analysis

Total RNA was isolated from epididymis epithelial cells using TRIzol reagent following the manufacturer's instructions. cDNA was synthesized from 1 μg of the total RNA using M-MLV Reverse Transcriptase in a total reaction volume of 25 μl. PCR amplification of the cDNA was performed with *Taq* polymerase and 1 μmol/l specific primers in separate PCR reactions. For TLR2, the protocol was 35 cycles of denaturation (95 °C for 45 s), annealing (56 °C for 45 s) and extension (72 °C for 1 min). For TLR4, the protocol was 35 cycles of denaturation (95 °C for 45 s), annealing (54 °C for 45 s), and extension (72 °C for 1 min). For nitric oxide synthase (iNOS), the protocol was 35 cycles of denaturation (95 °C for 45 s), annealing (63 °C for