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Original article

Evidence for *Gardnerella vaginalis* uptake and internalization by squamous vaginal epithelial cells: implications for the pathogenesis of bacterial vaginosis

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Abstract

Bacterial vaginosis (BV), a common condition seen in premenopausal women, is associated with preterm labor, pelvic inflammatory disease, and delivery of low birth weight infants. *Gardnerella vaginalis* is the predominant bacterial species associated with BV, although its exact role in the pathology of BV is unknown. Using immunofluorescence, confocal and transmission electron microscopy, we found that VK2 vaginal epithelial cells take up *G. vaginalis* after exposure to the bacteria. Confocal microscopy also indicated the presence of internalized *G. vaginalis* within vaginal epithelial cells obtained from a subject with BV. Using VK2 cells and ^{35}S labeled bacteria in an invasion assay, we found that a 1 h uptake of *G. vaginalis* was 21.8-fold higher than heat-killed *G. vaginalis*, 84-fold compared to *Lactobacillus acidophilus* and 6.6-fold compared to *Lactobacillus crispatus*. Internalization was inhibited by pre-exposure of cells to cytochalasin-D. In addition, the cytoskeletal protein vimentin was upregulated in VK2 cells exposed to *G. vaginalis*, but there was no change in actin cytoskeletal polymerization/rearrangements or vimentin subcellular relocalization post exposure. Cytoskeletal protein modifications could represent a potential mechanism for *G. vaginalis* mediated internalization by vaginal epithelial cells. Finally, understanding vaginal bacteria/host interactions will allow us to better understand the underlying mechanisms of BV pathogenesis.
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Keywords: Bacterial vaginosis; *Gardnerella vaginalis*; Internalization; Cytochalasin-D

1. Introduction

In women of child bearing age, bacterial vaginosis (BV) is the most common cause of vaginitis and has been associated with fetal loss, chorioamnionitis, cervicitis, endometritis, urinary tract infections, cervical intraepithelial neoplasia, pelvic inflammatory disease, preterm labor and delivery of low birth weight infants, as well as an increased risk for HIV-1 infection [1–9]. BV is characterized by the loss of vaginal lactobacilli usually found in healthy women and an overgrowth of anaerobes, including *Gardnerella vaginalis* and *Mycoplasma hominis*, as well as Mobiluncus, Bacteroides, Prevotella, and Peptostreptococcus species [10–14]. These events are triggered by mechanisms that are not well understood. Analysis of vaginal biopsies revealed that BV is characterized by a dense biofilm dominated by *G. vaginalis* and other fastidious anaerobes on the vaginal epithelium [10,15,16]. *G. vaginalis* alone is insufficient to cause BV in most cases, however this bacterial species is believed to be required for the occurrence of BV and is recovered from virtually all women with BV [17,18]. Recent studies suggest that *G. vaginalis* is the predominant anaerobe found in BV and is more virulent than other BV associated anaerobes [19]. In an

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analysis of vaginal adherence, the ability to form biofilms and cytotoxicity, *G. vaginalis* was shown to have a greater potential for virulence than other BV associated anaerobes [19]. It was suggested that other BV associated bacteria represent avirulent opportunists that colonize the vagina after an initial infection by *G. vaginalis*. BV is also associated with a high rate of recurrence after standard metranidizole therapy and can be difficult to clinically manage [20]. Recurrent BV has been linked with persistence of *G. vaginalis* [21]. We therefore surmised that the uptake/internalization of *G. vaginalis* by vaginal epithelial cells may contribute to the high recurrence rate of BV.

We have taken a molecular approach in an attempt to understand how the physical association between *G. vaginalis* and the vaginal mucosa could contribute to its persistence on the vaginal epithelium and the pathogenesis of BV. We show *in vitro* and *in vivo* data that support uptake of *G. vaginalis* when exposed to vaginal epithelial cells (VEC), whether they are immortalized VK2 cells or cells obtained from human vaginal lavage samples. Moreover, using a ^{35}S labeling invasion assay, we found evidence of internalization of *G. vaginalis* and upregulation of the cytoskeletal protein vimentin. This is the first report showing uptake and internalization of *G. vaginalis* by vaginal epithelial cells. Thus, the results may offer new insights into the overall pathogenesis of BV.

2. Materials and methods

2.1. Cervicovaginal lavages

Cervicovaginal lavages and subsequent cell samples (CVL) were collected from OB-GYN patients attending clinics at Metro General Hospital in Nashville, TN. CVL specimens were procured using sterile saline following a Meharry Medical College IRB-approved protocol. Samples were designated as BV- or BV+according to Amsel criteria and gram stain results, using a nugent score of $\geq$7 [14,22]. Specimens were transported to the laboratory on ice for processing. CVL-VEC were examined by wet mount and stained with crystal violet for the presence of clue cells and then gram stained for confirmation of the diagnosis of BV. Smears of CVL-VECs from BV+ and BV− patients were prepared for immunofluorescence (IFA) staining and confocal microscopy.

2.2. Cultivation of the VK2 vaginal epithelial cells

Human immortalized VK2 cells (ATCC 2616), obtained from the American Type Culture Collection, were selected for use because these cells represent squamous vaginal epithelial cells that are sloughed off and contained in vaginal discharges used to clinically identify BV [23]. They represent cells lining the vaginal wall that are in direct contact with the vaginal microflora.VK2 cells were either maintained in keratinocyte serum-free complete media (KSFM) or calcium-free KSFM complete media prepared according to the manufacturer's instructions (Gibco, Grand Island, NY). Cells were passaged by trypsinization and fresh medium was added every three days during cultivations.

2.3. Laboratory cultivation of G. vaginalis, inoculum preparation, and antibody identification

G. vaginalis bacteria ATCC (14018) was obtained from the American Type Culture Collection (Rockville, MD). Cultivation of *G. vaginalis* was performed using ATCC medium 1685 (NYC III medium). Broth cultures were inoculated with 250 µl of frozen stock bacterial cultures in 1.37% proteose peptone and 6.7% glycerol (stored at −80 °C). Broth cultures were cultivated at 37 °C in an anaerobic chamber using a GasPak system. Bacterial pellets were resuspended in supplemented KSFM complete media. Resuspended cells were quantitated by optical density at OD_{600} nm. Bacterial suspensions were used to inoculate VK2 cells at 0.34 OD_{600}/ml, which is equivalent to a multiplicity of infection between 10 and 50. *G. vaginalis* was heat-killed at 80 °C for 20 min in a waterbath.

A specific monoclonal antibody from AbD Serotec (Kidlington, UK) that recognizes a 62 KDa surface protein was used to detect *G. vaginalis* in VK2 cells and smears from BV+ CVLs by IFA. This antibody was also used to detect *G. vaginalis* in VK2 cells exposed to bacteria that were also dual labeled for estrogen receptor beta.

2.4. Immunofluorescence

Chamber slides containing monolayers of VK2 cells at a density of 2.5×10^5 cells/well were exposed to *G. vaginalis* for 24 h along with control cultures without bacteria. Cells were washed twice with phosphate buffer saline pH 7.4 (PBS), air dried, and fixed in absolute methanol at −20 °C for 10 min. Cells were then air dried for 15 min, hydrated in Tris saline (50 mM Tris, 150 mM NaCl) pH 7.4 for 5 min and incubated at 37 °C for 1 h with a mixture of a rabbit polyclonal antibody to estrogen receptor beta at 1:100 dilution (Millipore Billerica, MA) and a monoclonal antibody to a surface protein (described above) used to identify *G. vaginalis* at 1:50 dilution. Cells were then washed 3 times with Tris saline and incubated at 37 °C for 30 min with a mixture of secondary donkey anti-rabbit antibodies conjugated to rhodamine, and donkey anti-mouse antibodies conjugated to FITC (Jackson ImmunoResearch, West Grove, PA), both at a 1:100 dilution in PBS (pH 7.4). The cells were then washed in Tris saline and mounted with Vectashield mounting media containing 1.5 µg/ml of DAPI (Vector Labs, Burlingame, CA). Fluorescent images were observed and photographed with a Nikon TE-2000S fluorescent microscope mounted with a CCD camera.

2.5. Confocal and electron microscopy

For confocal microscopy, dual labeled cells stained by immunofluorescence were examined with a Nikon TE2000-U C1 laser scanning confocal microscope with EZC1 2.30 software. Serial Z-sections of 0.05 µm intervals were examined for evidence of bacterial internalization at an excitation of

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