Expression and Function of Bactericidal/Permeability-Increasing Protein in Human Genital Tract Epithelial Cells

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Genital tract epithelia regularly encounter and adapt to the existence of bacterial pathogens. This study provides evidence that the endocervical and ectocervical epithelia of the human female genital tract express bactericidal/permeability-increasing protein (BPI). The constitutive expression of BPI was restricted to cell-bound protein and unaffected by human papillomavirus type 16/E6E7 immortalization and pro-inflammatory cytokine stimulation. Epithelial BPI was, in part, responsible for killing a commensal strain of Escherichia coli. The results of the present study suggest that BPI is tightly regulated and functionally expressed by epithelial cells in the female reproductive tract and may play a role in regulating bacterial colonization in the genital mucosa.

The mucosal lining of the human female genital tract consists of morphologically and functionally distinct compartments that have evolved multiple mechanisms to tolerate commensal microflora while providing defense against pathogenic microorganisms. The single-layer columnar epithelium of the endocervix is relatively protected from ascending vaginal microflora by the cervical mucus and provides the conduit to the endometrium, which is the upper, sterile compartment of the uterine mucosa. The stratified nonkeratinizing squamous epithelia of the vagina and the ectocervix are normally colonized by a complex mixture of gram-positive and gram-negative microorganisms and are periodically exposed to nonresident microorganisms during sexual intercourse and to blood during menses. These exposures represent unique challenges that, along with sensitivity to regulation by sex hormones, distinguish the genital tract epithelia from any other mucosal compartment.

Mucosal epithelial cells harbor a number of antimicrobial factors that form a biochemical barrier to microbial colonization and that are critical to the maintenance of host-microbe homeostasis at the mucosal surface [1]. Among these factors is bactericidal/permeability-increasing protein (BPI), which has recently been reported for the first time in human mucosal epithelia of the digestive tract [2] but which, to our knowledge, has not been studied in genital tract epithelia until now. We used normal tissues and cell lines to determine the cellular localization and to explore the functionality of epithelial BPI in the human uterine cervix.

Materials and methods. Human cervical tissues were obtained from hysterectomies for benign conditions performed at Brigham and Women’s Hospital, in accordance with institutional review board–approved protocol. Previously established, immortalized ectocervical (Ect1/E6E7) and endocervical (End1/E6E7) epithelial cell lines were grown in keratinocyte serum-free medium (Invitrogen), as described elsewhere [3]. In vitro reconstructed human ectocervical epithelial equivalents (VEC-100), in 24-well plate–size inserts, were purchased from MatTek. Caco-2 intestinal epithelial cells were obtained from the American Type Culture Collection and cultured in Dulbecco’s MEM supplemented with 10% fetal bovine serum, in addition to 1% penicillin and streptomycin (GIBCO/BRL Life Technologies). Recombinant human cytokines interleukin (IL)–1β, interferon (IFN)–γ, and tumor necrosis factor (TNF)–α were obtained from R&D Systems.

Immunostaining of epithelial cells or tissues was performed using an alkaline phosphatase biotin-streptavidin amplified system (StrAviGen; BioGenex) and the Fast Red Substrate System (Dako), as described elsewhere [3]. For these experiments, cells were grown in 8-chamber tissue-culture slides (BD Falcon), fixed in absolute ethanol for 20 min at 4°C, and kept at −80°C until stained. Tissues were deparaffinized in xylene and ethanol, and antigen retrieval was performed in an EDTA-based EZ-AR2 solution, by use of an EZ-Retriever oven (Biogenex), in accordance with the manufacturer’s instructions.

The following anti-BPI antibodies were used: (1) affinity-
purified rabbit anti–human BPI polyclonal antibody (PAb) [4], (2) rabbit anti–human BPI PAb (XOMA), and (3) anti–human BPI mouse monoclonal antibody (MAb) clone 3F9 (HyCult Biotechnology). To determine specificity, the 2 anti–BPI PAbs were adsorbed with excess recombinant human BPI (XOMA). Microscopy was performed using an Olympus BX60 microscope and a Q-color 3 digital camera (Olympus America) with QCapture software (version 1.71.0; Quantitative Imaging).

For transcriptional analysis, epithelial cells in 6-well plates and VEC-100 tissues in 24-well plate inserts were stimulated with TNF-α (20 ng/mL), IL-1β (20 ng/mL), and IFN-γ (300 U/mL) in a 5% CO2 incubator at 37°C for 6 h. RNA was isolated using an RNeasy Protect minikit (Qiagen), in accordance with the manufacturer’s instructions. Reverse transcription (RT) was performed using the Iscript kit from Bio-Rad. RT–polymerase chain reaction (PCR) analysis was performed using the following primer sets (Invitrogen): human BPI forward (5′-GCACCTGTTCCTGATGGG-3′) and reverse (5′-TTTCTTG-3′) primers, yielding a 256-bp product; and human β-actin forward (5′-TGACCCAGATCATGTGGAGA-3′) and reverse (5′-AGTCATTACAGATGCAGTGAGC-3′) primers, yielding a 131-bp product. The PCR contained, in addition to 2 μg DNA, 1 μmol each of the forward primer and the reverse primer, 10 μL of 5X PCR buffer, 1 mmol/L MgSO₄, 0.2 mmol/L dNTP, and 5 U of Taq-enzyme mix in a total volume of 50 μL. Each primer set was amplified using 25–30 cycles of 94°C for 45 s, 60°C for 45 s, 72°C for 45 s, and a final extension of 72°C for 5 min. PCR products were then visualized on a 1.5% agarose gel containing 0.5 μg/mL ethidium bromide.

For protein analysis, epithelial cells and VEC-100 tissues were lysed in 25 mmol/L Tris buffer with 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, 1% (vol/vol) IGEPAL CA-630, proteinase inhibitor cocktail, and 0.4 mmol/L phenylmethylsulfonyl fluoride (all from Sigma-Aldrich). The lysates were cleared of cellular debris by centrifugation at 10,000 g for 10 min. Total protein was determined by bicinchonic acid assay (Pierce).

Human BPI ELISA (HyCult) and IL-6 QuantiGlo ELISA (R&D Systems) were performed, in accordance with the manufacturer’s instructions, using a Victor2 Multilabel reader (Perkin-Elmer Life Sciences). The BPI levels were measured in cell-culture supernatants and in cell lysates.

Cell lysates (2.5 mg of total protein) were mixed with 5 μg of anti-BPI antibody (gift from XOMA) or control rabbit IgG (Santa Cruz Biotechnology) and 80 μL of protein A beads (Amersham Biosciences), and the mixture was then rotated overnight at 4°C. The beads were washed; a sample buffer containing 50% glycerol, 0.25 mol/L Tris (pH 6.8), 2.5% SDS, and bromphenol blue was added; and samples were resolved on a 12% polyacrylamide gel. Proteins were subsequently transferred onto nitrocellulose and probed with anti-human BPI mouse MAb clone 3F9 (HyCult Biotechnology) at a concentration of 1 μg/mL. After incubation with an anti-mouse secondary antibody (MP Biomedicals) and washing, proteins were visualized using enhanced chemiluminescence (KPL).

For bacterial killing assays, Ect1/E6E7 and End1/E6E7 were

Figure 1. Bactericidal/permeability-increasing protein (BPI) expression in human cervical epithelium. Immunohistochemical localization (red) of BPI in ectocervical tissue (A and B), squamous epithelium (VEC-100) reconstructed ex vivo from primary ectocervical epithelial cells (C), endocervical epithelial tissue (E), and immortalized endocervical (D) and endocervical (F and G) cell lines. Arrows indicate mitotic cells. Specific immunostaining was completely abolished by absorption with recombinant human BPI (B and G). H, Reverse transcription–polymerase chain reaction in endocervical End1/E6E7 (lane 1) and ectocervical Ect1/E6E7 (lane 2) cells, representing 1 of 3 experiments. J, BPI demonstrated by immunoprecipitation with BPI-specific antibody and Western blot in endocervical (lane 2) and ectocervical (lane 3) cells. Recombinant BPI (300 ng) (lane 1) and the sample obtained from incubating the control antibody with lysates from ectocervical cells (lane 4) served as respective positive and negative controls. Results are 1 of 2 independent experiments. J, BPI levels in ectocervical (Ect1/E6E7) epithelial cell lysates and interleukin (IL)–6 levels in supernatants from the same cultures, determined by ELISA after 24 h of stimulation with tumor necrosis factor (TNF)-α. Similar results were obtained with the End1/E6E7 epithelial cells. Data are mean ± SE results from 1 of 3 independent experiments with each cell line. (Original magnification, ×10 (A–C) and ×20 (D–G)
Figure 2. Killing of a commensal strain of *Escherichia coli*, mediated by endocervical (End) and ectocervical (Ect) epithelial cells over a period of 30 min. Significant reductions in colony-forming unit (cfu) counts in bacterial-epithelial coculture are presented as the percentage change from the average in bacterial culture alone. Values are mean ± SE results from 6 measurements of duplicate cultures in 1 of 3 independent experiments with each cell line (A). Furthermore, colony-forming units were moderately but significantly higher in End (B) and Ect (C) epithelial-bacterial cocultures exposed to bactericidal/permeability-increasing protein (BPI)–neutralizing antibody, compared with culture in the presence of the same antibody adsorbed with recombinant human BPI. Similar data (not shown) were obtained at the 15- and 45-min time points. Data are expressed as mean ± SE results of 6 measurements in 1 of 4 independent experiments. *P < .01; **P < .001.

grown in 6-well cell-culture plates (Falcon) to confluence and washed with Hanks’ balanced salt solution (Sigma-Aldrich) before bacteria were added. A commensal strain, HS-4 of *Escherichia coli* (cited in [5]), grown to late log phase in Luria-Bertani broth, was added to epithelial monolayers at a ratio of 10–20:1 bacteria per adherent epithelial cell and allowed to incubate for 15, 30, and 45 min at 37°C in Hanks’ balanced salt solution. To assess the contribution of BPI to the killing of bacteria, 10 μg of either a BPI-neutralizing antibody (HyCult) or a control preadsorbed antibody was used to pretreat the cells for 30 min before adding bacteria. After incubation with bacteria, supernatants and cells were collected and hypotonically lysed together in ice-cold water. These lysates were diluted and plated on Luria-Bertani agar (Becton Dickinson), and colony-forming units were counted the following day. Controls consisted of bacteria alone that were maintained under conditions identical to those used for the bacterial-epithelial cocultures and then plated out at the relevant time point. Results were calculated as percentage of bacteria killed, by use the following formula: \[ \frac{(\text{colony-forming units of bacteria alone} - \text{colony-forming units of bacterial-epithelial coculture})}{\text{bacteria alone}} \times 100. \]

GraphPad Prism (version 4.0; GraphPad Software) was used for statistical analysis. Paired Student’s *t* tests were used to compare baseline versus treated samples. Analysis of variance with Bonferroni posttest was used for multiple comparisons.

**Results.** Immunohistochemical analysis of normal human female genital tract tissues (*n* = 8) revealed that BPI was abundantly expressed by the squamous ectocervical epithelium (figure 1A) as well as by the columnar endocervical epithelium (figure 1E). Similarly, BPI was abundantly expressed by the ex vivo reconstructed ectocervical epithelium (figure 1C). The ectocervical (figure 1D) and endocervical (figure 1F) immortalized epithelial cell lines showed similar levels of BPI-specific staining with all antibodies tested in this study. BPI staining appeared stronger in the perinuclear area, suggesting that BPI is present in the cytoplasm. Interestingly, higher BPI-staining intensity was consistently observed in mitotic cells in both en-
docervical and ectocervical cell lines. The specificity of the BPI staining was confirmed by negative staining patterns observed with the adsorbed BPI antibodies (figure 1B and 1G).

BPI mRNA was detected in resting endocervical and ectocervical epithelial cells in 3 independent experiments (figure 1H). No consistent increase in BPI mRNA levels was seen in response to stimulation with IL-1β, TNF-α, and IFN-γ in these experiments (data not shown). Although BPI was undetectable in cell-culture supernatants (data not shown), cell-bound BPI was detected in cell lysates from immortalized endocervical and ectocervical cells by use of immunoprecipitation (figure 1J) and ELISA (figure 1J). Similar results were obtained using the VEC-100 tissues generated from primary cervical epithelial cells (data not shown). The BPI levels in cell lysates were not significantly affected by stimulation with TNF-α at doses effective at inducing proinflammatory signaling as measured by IL-6 production (figure 1J).

An assay designed for assessment of bactericidal properties of adherent epithelial cells over a time course of 45 min (cited in [2, 6]) demonstrated that both endocervical and ectocervical cells killed a commensal E. coli strain and that this effect was at least partly the result of BPI, because the effect was diminished in the presence of a BPI-neutralizing antibody, compared with control BPI-adsorbed antibody (figure 2). Both cell lines mediated killing of bacteria of a similar magnitude in the presence of control antibody, with endocervical cells resulting in means ± SEs of 86% ± 5%, 85% ± 8%, and 77% ± 8% of bacteria killed and the ectocervical cells resulting in means ± SEs of 80% ± 3%, 79% ± 3%, and 80% ± 5% of bacteria killed, at 15, 30, and 45 min, respectively. The BPI-neutralizing antibody inhibited the killing of bacteria moderately but significantly (P < .05) across all time points, with the killing of bacteria inhibited by means ± SEs of 11.2% ± 0.1%, 9.9% ± 1.5%, and 12.8% ± 4% with endocervical cells and by means ± SEs of 20.1% ± 8%, 19.7% ± 1%, and 22.5 ± 2.5% with ectocervical cells, respectively, compared with control adsorbed antibody.

Discussion. BPI, also known as the “cationic antibacterial protein of 57 kDa” (CAP57) and the “bactericidal protein of 55 kDa molecular mass” (BP55), has been studied in cells of myeloid lineage [7] for 3 decades and has only recently been reported in mucosal epithelial cells [2]. BPI is especially abundant in the azurophilic granules of human neutrophils, accounting for most of their bactericidal properties because of its high-affinity binding with the structurally conserved lipid A region of lipopolysaccharide (LPS) on gram-negative organisms [8, 9]. In contrast, BPI appeared to be responsible for only 10%–20% of the killing of gram-negative bacteria in our cervical epithelial model system, which is consistent with the relatively low expression levels of epithelial BPI, compared with those of neutrophil BPI [6], and the production of multiple other bactericidal factors by the epithelial cells [1].

Another important implication of high-affinity interaction of BPI with lipid A is the neutralization of bacterial endotoxin. BPI released by neutrophils is capable of inhibiting the LPS-dependent inflammatory responses in monocytes and macrophages by competing with the circulating LPS-binding protein, thus preventing its association with CD14 and Toll-like receptor (TLR) 4 [4, 10]. The BPI-TLR interaction in the epithelial cell context is still unknown. Human cervical epithelial cells are capable of responding to gram-negative ligands in a TLR4-independent manner, limiting the role that BPI plays in this process [11–13]. Under culture conditions, these cells are TLR4− [11]; however, host or environmental factors may modulate TLR repertoires in vivo, as indicated by the differential TLR4 deficiency in genital tract epithelial tissues [14, 15]. On the other hand, soluble TLR4 has been demonstrated in the endocervical canal, suggesting yet another BPI-bypassing mechanism of LPS neutralization [14].

The results of the present study demonstrate that both columnar and stratified nonkeratinized cervical epithelia cell types constitutively express BPI, which is unchanged by human papillomavirus type 16/E6E7 immortalization or by proinflammatory cytokines. Considered together, our findings suggest that BPI is tightly regulated at the mucosal surface and may have an important housekeeping function in these epithelia. The stronger expression of BPI by mitotic cells suggests that epithelial BPI may serve to prevent gram-negative bacterial invasion in areas of epithelial damage and intensive tissue repair. Additional studies are needed to clarify the role that epithelial BPI plays in the innate immunity of the female reproductive tract.

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