Human Epididymis Protein 4 and Secretory Leukocyte Protease Inhibitor in Vaginal Fluid: Relation to Vaginal Components and Bacterial Composition

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Abstract

Human epididymis protein 4 (HE4) is a protease inhibitor and a recently identified serum biomarker for ovarian cancer. Properties of HE4 in the genital tract of healthy women have not been evaluated. We evaluated associations between HE4 and a second vaginal protease inhibitor, secretory leukocyte protease inhibitor (SLPI), with vaginal concentrations of innate immune mediators or proteases and with the types of vaginal bacterial communities. Vaginal secretions were collected from 18 healthy reproductive age women and assayed by enzyme-linked immunosorbent assay for concentrations of HE4, SLPI, kallikrein 5, cathepsin B, interleukin 1 β (IL-1 β), IL-1 receptor antagonist (IL-1ra), mannose-binding lectin (MBL), the inducible 70-kDa heat shock protein, and matrix metalloproteinase (MMP)-8. The species composition of vaginal bacterial communities in 16 women was characterized by sequencing amplicons derived from 16S bacterial ribosomal RNA genes. Correlations between any 2 assays were analyzed by the Spearman rank correlation tests. Differences in the concentrations of HE4 and SLPI, and between soluble components and vaginal community types, were analyzed by the Mann-Whitney *U* tests. Vaginal HE4 concentrations, but not SLPI levels, were positively correlated with levels of IL-1 β ($P = .0152$), IL-1ra ($P = .0061$), MBL ($P = .0100$), and MMP-8 ($P = .0315$). The median vaginal HE4 level, as well as concentrations of MBL, IL-1 β , IL-1ra, and MMP-8, was highest when *Gardnerella vaginalis* dominated a vaginal community. The association between HE4, elevated levels of proteases, immune mediators and high proportions of *G vaginalis* strongly suggests that HE4 is a component of the proinflammatory immune response in the female genital tract.

Keywords

human epididymis protein 4, secretory leukocyte protease inhibitor, vagina, vaginal microbiome, cytokines

Introduction

Human epididymis protein 4 (HE4) has drawn considerable interest due to its clinical value as a serum biomarker for ovarian cancer.¹ It is highly expressed in serous and endometrioid ovarian cancer cells but absent in normal ovarian epithelium.^{2,3} The expression of HE4 is also elevated in endometrial^{3,4} and lung cancer³ (although not as prominent as in ovarian cancer) and detectable at lower levels in several other malignancies.³ In nonmalignant tissues, HE4 is highly expressed in fibrosis-associated myelofibroblasts,⁵ the human epididymis,⁶ epithelia of the female genital tract, respiratory tract, and salivary gland.³

The *HE4* gene is located in a region that codes for several proteins that contain whey acidic protein (WAP) domains. Its location on chromosome 20q13 is known as the WAP 4-disulfide core domain (WFDC) locus.⁷ HE4 is also known as WFDC-2. The most well-characterized member of this family is secretory leukocyte protease inhibitor (SLPI).⁸ This serine protease inhibitor is produced in glandular epithelial

cells in the endometrium and decidua⁹ and is believed to possess antibacterial and antiinflammatory properties.⁹⁻¹¹ A recent study has clearly demonstrated that HE4 is also a protease inhibitor with a specificity that differed from SLPI. HE4 and SLPI share the ability to inhibit multiple serine proteases, whereas HE4 is also active against aspartyl and cysteine proteases. In addition, SLPI is a monomeric protein, while HE4 exists as a disulfide bonded trimer.¹²

To explore the possible functions of HE4 and SLPI in the female genital tract, we analyzed vaginal secretions for

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associations between HE4 and SLPI concentrations and a number of vaginal components active in innate immune or proteolytic activities. The relationship between vaginal levels of HE4, SLPI, and types of bacterial communities in the vagina was also assessed.

Materials and Methods

The study population comprised 18 white women of reproductive age seen for their annual routine physical examination. All were without any clinical signs or symptoms of infection, were not pregnant, had not used antibiotics for at least 4 weeks, and refrained from sexual intercourse for at least 48 hours. The study was approved by the institutional review board of Weill Cornell Medical College, and all participants gave written informed consent.

During their gynecological examination, a cotton swab was used to collect secretions from the posterior vagina. The swab was removed and vigorously shaken into 1 mL of sterile phosphate-buffered saline. The sample was centrifuged to remove cells and particulate material, and aliquots of the supernatant were frozen at -80°C until they were analyzed. In 16 women, an additional vaginal sample was obtained using the Copan ESwarbs Sample Collection System (Fisher Scientific, Pittsburgh, Pennsylvania). Samples were frozen at -80°C until shipped to the University of Idaho. The composition of vaginal bacterial communities was determined by pyrosequencing and classifying the V1 to V3 region of bacterial 16S ribosomal RNA gene sequences as described previously.¹³

Commercial enzyme-linked immunosorbent assay kits were used to determine concentrations of various analytes in vaginal supernatants. The lower limit of sensitivity and source of each kit were as follows: HE4 38 ng/mL, SLPI 25 pg/mL, interleukin 1 β (IL-1) 3.9 pg/mL, IL-1 receptor antagonist (IL-1ra) 39 pg/mL, mannose-binding lectin (MBL) 15.8 pg/mL, the inducible 70-kDa heat shock protein (hsp70) 156 pg/mL, metalloproteinase 8 (MMP-8) 62.5 pg/mL, kallikrein 5 78 pg/mL, and cathepsin B 5 pg/mL. The kits used to assay HE4 and cathepsin B were from Antibodies-online (Atlanta, Georgia) whereas all others were from R&D Systems (Minneapolis, Minnesota). For each assay, the values were converted to ng/mL or pg/mL by comparison to a standard curve that was generated in parallel to each assay.

Associations between concentrations of HE4 or SLPI and the other compounds were evaluated by the Spearman rank correlation test. Differences in concentration between HE4 and SLPI, and between HE4 or SLPI and vaginal bacterial community composition, were determined by Fisher exact test. A P value $<.05$ was considered significant.

Results

The concentrations of each of the tested compounds in vaginal secretions are shown in Table 1. Every sample had detectable levels of HE4, SLPI, IL-1ra, MMP-8, kallikrein 5, and cathepsin B; 5 samples were negative for IL-1 β and 3 each were

Table 1. Concentrations of Soluble Mediators in Vaginal Secretions.^a

Mediator	Median	Range
HE4	1400 ng/mL	33-5530 ng/mL
SLPI	156 ng/mL ^b	1.3-715 ng/mL
Cathepsin B	18.1 ng/mL	0.8-65.8 ng/mL
Kallikrein 5	2.2 ng/mL	0.4-20.3 ng/mL
IL-1 β	49.1 pg/mL	<3.9-1074 ng/mL
IL-1ra	7.0 ng/mL	0.8-64.3 ng/mL
Mannose-binding lectin	80.0 pg/mL	<15.8-149 pg/mL
70-kDa heat shock protein	1.5 ng/mL	<0.2-63.0 ng/mL
MMP-8	19.1 ng/mL	0.3-50.5 ng/mL

Abbreviations: HE4, human epididymis protein 4; SLPI, secretory leukocyte protease inhibitor; IL, interleukin; ra, receptor antagonist; MMP, matrix metalloproteinase.

^aVaginal secretions from 18 healthy reproductive age women were tested for immune mediator concentrations by enzyme-linked immunosorbent assay.

^b $P \leq .0001$ SLPI versus HE4.

Table 2. Associations Between Concentrations of HE4 or SLPI and Immune Mediators in Vaginal Secretions.^a

Mediator	P Value	
	HE4	SLPI
HE4		.7416
Cathepsin B	.0068	.2502
Kallikrein 5	.0497	.7170
Interleukin-1 β	.0152	.4004
IL-1ra	.0061	.1182
Mannose-binding lectin	.0100	.6862
70 kDa heat shock protein	.1871	.0579
MMP-8	.0315	.0800

Abbreviations: HE4, human epididymis protein-4; SLPI, secretory leukocyte protease inhibitor; IL-1ra, interleukin-1 receptor antagonist; MMP, matrix metalloproteinase

^aConcentrations of immune mediators in vaginal secretions from 18 healthy reproductive age women were tested for associations with HE4 and SLPI concentrations by Spearman rank correlation test. Positive correlations are in boldface.

negative for MBL and hsp70. The vaginal concentration of HE4 exceeded that of SLPI ($P < .0001$).

Associations between concentrations of HE4 or SLPI and the other soluble vaginal components are shown in Table 2. The HE4 levels were highly and positively correlated with concentrations of IL-1 β ($P = .0152$), IL-1ra ($P = .0061$), MBL ($P = .0100$), MMP-8 ($P = .0315$), cathepsin B ($P = .0068$) and marginally correlated with kallikrein 5 ($P = .0497$). In marked contrast, there was no relation between SLPI levels and any of these compounds. In addition, there were no associations between concentrations of HE4 and SLPI ($P = .7416$).

The analysis of vaginal microbiomes revealed that *Lactobacillus crispatus* dominated the communities of 8 women, while *Gardnerella vaginalis* and *Lactobacillus iners* were dominant in 6 and 2 women, respectively. The relationships between the predominant bacterial species and concentrations of soluble vaginal compounds are shown in Table 3. Median

Table 3. Association Between Predominant Vaginal Bacteria and Concentration of Soluble Vaginal Mediators.^a

Mediator	Median (range)		
	<i>Lactobacillus crispatus</i> , N = 8	<i>Lactobacillus iners</i> , N = 2	<i>Gardnerella vaginalis</i> , N = 6
HE4, ng/mL	740 (8-1400) ^b	5180 (4830-5550)	3930 (610-4830)
SLPI, ng/mL	551 (150-715)	164 (163-165)	173 (96-470)
Cathepsin B, ng/mL	14.2 (3.9-28.8)	26.1 (20.1-32.1)	31.8 (12.1-65.8)
Kallikrein 5, ng/mL	0.9 (0.4-4.6)	13.5 (6.7-20.3)	2.2 (1.1-6.9)
Interleukin-1 β , pg/mL	4.5 (<3.9-90.7) ^c	538 (<3.9-737)	134 (29.3-533)
IL-1ra	2.6 (0.8-7.3) ^d	31.6 (26.9-36.8)	22.4 (2.4-64.3)
Mannose-binding lectin, pg/mL	7.0 (< 15.8-1272) ^e	368 (159-577)	187 (19.3-874)
70-kDa heat shock protein, pg/mL	0.2 (0.2-1.7)	9.4 (2.4-16.4)	5.0 (0.2-8.7)
MMP-8	9.7 (0.3-35.2) ^f	20.5 (9.0-31.9)	30.2 (12.0-50.5)

Abbreviations: HE4, human epididymis protein-4; SLPI, secretory leukocyte protease inhibitor; IL-1ra, interleukin 1 receptor antagonist; MMP-8, matrix metalloproteinase-8.

^aThe vaginal microbiomes in 16 women were determined by gene amplification of the gene coding for 16S bacterial ribosomal RNA. The biotype present at the highest frequency in each woman is delineated above.

^bP = .0101 versus *G vaginalis*, .0444 versus *L iners*.

^cP = .0224 versus *G vaginalis*, .0556 versus *L iners*.

^dP = .0293 versus *L crispatus*.

^eP = .0373 versus *G vaginalis*.

^fP = .0293 versus *G vaginalis*.

concentrations of HE4 were higher and similar when *L iners* or *G vaginalis* were dominant members of vaginal communities when compared to communities in which *L crispatus* predominated ($P = .0101$, *L crispatus* vs *G vaginalis*). In contrast, median levels of SLPI were approximately 3-fold higher when *L crispatus* was dominant although this difference was not statistically significant ($P = .0952$, *L crispatus* versus *G vaginalis*). The median IL-1 β ($P = .0224$), IL-1ra ($P = .0293$), MBL ($P = .0373$), and MMP-8 ($P = .0293$) levels were all greatly elevated when *G vaginalis* was dominant as opposed to when communities were dominated by *L crispatus*.

Discussion

Both HE4 and SLPI were identified as components of vaginal secretions from each of 18 reproductive age women investigated. However, there were strikingly different associations between concentrations of each protease inhibitor and other components of the vaginal milieu. Although HE4 levels were positively correlated with vaginal concentrations of IL-1 β , IL-1ra, MBL, MMP-8, and cathepsin, none of these compounds were related to the concentration of SLPI.

The combined data suggest that HE4, but not SLPI, might be induced in response to vaginal communities dominated by certain bacterial species. IL-1 β is a proinflammatory cytokine that is released from macrophages and epithelial cells in response to infection,¹⁴ while IL-1ra is a competitive inhibitor of IL-1 β that is produced in response to elevated concentrations of IL-1 β to prevent excessive proinflammatory effects.¹⁵ MBL is a component of the innate immune system active against numerous viral, bacterial, and fungal infections¹⁶ and levels of MBL increase in response to an infectious event.¹⁷ MMP-8 is a member of the matrix metalloproteinase family that degrades

the extracellular matrix under adverse environmental conditions or during morphological changes.¹⁸ Levels of this protein are increased in the vaginas of women with bacterial vaginosis, and it reduces the integrity of the cervical plug.¹⁹ Cathepsin is a cysteine protease that contributes to degradation of the extracellular matrix²⁰ and is also involved in the maturation and release of biologically active IL-1 β .²¹ Kallikrein 5 is a serine protease found in cervicovaginal fluid and in supernatants from vaginal epithelial cell cultures. It also contributes to the remodeling of the cervical mucus.²² In contrast to the other compounds tested, the hsp70 concentration was apparently unaffected in response to changes in vaginal immune mediators. The extracellular level of this protein typically increases in response to a proinflammatory event.²³

The nature of the vaginal microbiota appears to influence the concentration of HE4 in the vagina. *Gardnerella vaginalis* often occurs as the dominant bacterial species in women diagnosed with bacterial vaginosis, a condition characterized by increased susceptibility to vaginal as well as upper genital tract infections.^{24,25} In communities dominated by *G vaginalis*, the level of HE4 is markedly elevated compared to its concentration when *L crispatus* predominates. A vaginal microbiota dominated by *L crispatus* is associated with vaginal health.^{13,26} Although *G vaginalis* is sometimes identified as the dominant member of vaginal communities in apparently healthy women,^{13,26} it is most commonly associated with a fluctuating or unstable vaginal bacterial milieu.

Only 2 of our patients had a vaginal microbiota in which *L iners* predominated, so any discussion of the related findings in these women must be viewed as preliminary. Although *L iners* is frequently the most abundant *Lactobacillus* phylotype in the vagina of apparently healthy women,^{13,26} it has also been associated with increased susceptibility to develop bacterial vaginosis.²⁷ The observation that vaginal levels of HE4, IL-1 β ,

IL-1ra, MBL, and MMP-8 were comparable when vaginal communities were dominated by *L iners* or *G vaginalis* suggests that this *Lactobacillus* species can induce a local host antibacterial immune response under certain circumstances. Further studies on a much larger cohort of women with vaginal communities dominated by *L iners* are required to validate this preliminary observation.

In contrast to the findings with HE4, the level of SLPI is elevated when *L crispatus* is present at high concentrations in the vagina and reduced when *G vaginalis* or *L iners* is dominant. The biological explanation for this occurrence remains to be determined. Perhaps different *Lactobacillus* species may preferentially induce specific protease inhibitors. Alternatively, *L iners* and *G vaginalis*, but not *L crispatus*, may release enzymes that are capable of degrading SLPI while having no negative effect on HE4 levels. The relationship between the differential production of HE4 and SLPI and the vaginal microbiota deserves further exploration.

In conclusion, HE4 and SLPI in the vagina appear to be differentially regulated in response to the vaginal microbiota and to the concentrations of antimicrobial and proinflammatory mediators.

Authors' Note

T. Orfanelli, A. Jayaram, G. Doulaveris contributed to study design and execution and critical discussion. LJ Forney contributed to microbiome analysis, critical discussion, and manuscript revisions. WJ Ledger contributed in clinical data analysis and critical discussion. S. S. Witkin contributed to study design, data analysis, manuscript drafting, and critical discussion.

Declaration of Conflicting Interests

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