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Gene Expression, Immunolocalization, and Secretion of Human Defensin-5 in Human Female Reproductive Tract

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This study describes the novel localization of the antimicrobial peptide human intestinal defensin-5 (HD-5) in female genital tract epithelia. Using a 3′ rapid amplification of cDNA ends (RACE) protocol, HD-5 was cloned from a vaginal epithelial cell RNA preparation, and its identity was confirmed by sequencing. Tissue samples from multiple donors were subsequently screened for HD-5 expression by reverse transcription polymerase chain reaction. HD-5 message was invariantly expressed by normal vagina and ectocervix and inflamed fallopian tube, but variably expressed by normal endocervix, endometrium, and fallopian tube (60, 64, and 29% of specimens, respectively). Expression in endometrium was the highest during the early secretory phase of the menstrual cycle. Using immunohistochemistry and confocal microscopy, HD-5 peptide was localized in the upper half of the stratified squamous epithelium of the vagina and ectocervix, with the intensity of cellular staining increasing toward the lumen. In positive endocervix, endometrium, and fallopian tube specimens, HD-5 was located in apically oriented granules and on the apical surface of a proportion of columnar epithelial cells. Using Western blot analysis, secreted HD-5 was detected in cervicovaginal lavages, with the highest concentrations found during the secretory phase of the menstrual cycle. We hypothesize that HD-5 is an intrinsic component of the female urogenital innate immune defense system and that its expression may be modulated by hormonal and proinflammatory factors. ***(Am J Pathol 1998, 152:1247–1258)***

Epithelial cells that line the surfaces of the body constitute a mechanical, chemical, and microbiological impediment to pathogenic agents. In the female lower genital tract, the stratified squamous epithelium of the vagina and ectocervix provide a substantive cellular barrier to sexually transmitted organisms, and this is supported by an indigenous microflora, an acidic microenvironment (pH 3.5 to 5.0), and a physical barrier of mucus derived from endocervical epithelial cells.[1,2] Cervical mucus also creates a plug that provides a physical barrier to sperm and microbe entry into the uterine cavity and in addition is a medium for endocervical-derived secretory IgA and antibacterial molecules such as lactoferrin, lysozyme, and zinc.[1–5] The fallopian tubes and uterus are classically described as sterile sites, but microorganisms can ascend from the lower tract and cause pelvic inflammatory disease (PID). Host factors associated with susceptibility to PID include vaginitis and bacterial cervicitis, compromise of the cervical barrier during ovulation or menstruation, or intrauterine devices.[6,7] However, compromise of the cervical barrier rarely leads to upper tract infection, and there is evidence to suggest that the endometrial cavity of apparently healthy women can be transiently colonized by pathogens of low virulence.[8–10] We therefore hypothesize that the upper female genital tract has a powerful innate immune system that can maintain a predominantly aseptic state and protect the reproductive organs from the devastating sequelae of PID.

Defensins are small (<4 kd), cationic antimicrobial peptides that have a broad spectrum of activity that includes gram-positive and gram-negative bacteria,[11] *Chlamydia*,[12] fungi,[13] protozoa,[14] and enveloped viruses.[15] The defensins contain six cysteine residues forming three disulfide bridges and are synthesized as preproproteins that undergo sequential processing to the mature, biologically active, cationic peptide.[16] Depending on the spacing of the cysteine residues and the connectivity of the disulfide bridges, defensins are classified into two families, the α-defensins and the β-defensins.[17] The α-defensins were first isolated from cells of the myeloid

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lineage,[18,19] but a unique α-defensin called cryptdin was subsequently cloned from murine small intestine and localized to the Paneth cell population.[20] Two human Paneth cell homologues have also been identified and named human defensin-5 (HD-5)[21] and human defensin-6 (HD-6).[22] HD-5 transcripts have also recently been found in human chorion, endocervix, and endometrium.[23] Both cryptdin and recombinant HD-5 have demonstrated antimicrobial activity against a variety of gastrointestinal pathogens.[24,25] The β-defensins appear to be more widespread in their distribution and have been described in bovine lingual[26] and tracheal[27,28] epithelial cells and neutrophils[29] and in human skin,[30] plasma,[31] and epithelium,[32,33] including that of the urogenital tract,[34] kidney,[31,33,34] pancreas,[33] and salivary glands.[33]

The detection of HD-5 mRNA in endometrium and endocervix indicates that this α-defensin could play a role in the innate defense of the female genital tract.[23] This is substantiated by the present study, which describes the novel cloning and sequencing of HD-5 from the stratified squamous epithelium of the vagina, the subsequent screening and localization of HD-5 in fallopian tube, endometrium, endocervix, ectocervix, and vagina, and the identification of HD-5 peptide in cervicovaginal secretions.

Materials and Methods

Tissues

Fallopian tubes (n = 21), endometrium (n = 28), endocervix (n = 15), and ectocervix (n = 14) were collected as discard material from hysterectomies performed for suspected leiomyomas or endometriosis. Vaginal tissue (n = 10) was obtained from anterior/posterior vaginal repairs. All women were premenopausal and not taking female steroid hormones or hormonal antagonists. Histological dating of endometrium was used to obtain menstrual cycle data. All tissues were deemed normal on histological examination with the exception of four fallopian tubes that were classified as acutely and/or chronically inflamed. Tissue samples were processed within 1.5 hours of removal; endometrium and cervix were trimmed to remove the majority of underlying stroma, and connective tissue and fat were removed from fallopian tubes. Specimens were snap frozen in liquid nitrogen and stored at −70°C before RNA extraction. When larger specimens were available, a small piece of the tissue was fixed overnight in 10% formaldehyde (Ultrapure EM grade, Polysciences, Warrington, PA), dehydrated in a stepwise series of alcohols, steeped in xylene, and then infiltrated in paraffin at 50°C before embedding.[35]

One specimen of vaginal tissue was used to prepare an enriched preparation of epithelial cells for cDNA cloning. In brief, the tissue was washed twice with Hanks' balanced salt solution and then incubated in keratinocyte growth medium (Clonetics Corp., San Diego, CA) supplemented with 5 U/ml dispase type I (Collaborative Biomedical Products, Bedford, MA) for 1 hour at 37°C. The epithelial surface was gently scraped to free sheets of epithelial cells, the cell-rich medium was collected and centrifuged, and the resulting cell pellet was solubilized in TriReagent (Molecular Research Center, Cincinnati, OH).

cDNA Cloning and Sequencing

A 3′ rapid amplification of cDNA ends (RACE) protocol was adapted to clone defensin cDNA from vaginal epithelial cells.[28] One microgram of total RNA was used to synthesize the first-strand cDNA with Superscript reverse transcriptase (BRL, Gaithersburg, MD). The reaction was performed in 5 μl using the adapter primer AP (5′-GGCCACGCGTCGACTAGTAC$(T)_{17}$-3′) at 42°C with the buffer supplied. After 30 minutes of incubation, 2 μl of the cDNA mixture was transferred to 48 μl of polymerase chain reaction (PCR) mixture containing 20 mmol/L Tris/HCl (pH 8.4), 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 200 mmol/L dNTP, 0.1 U/ml *Taq* DNA polymerase (Perkin-Elmer Cetus, Branchburg, NJ), 10 mCi of [α-^{32}P]dCTP (3000 Ci/mmol; Du Pont, Wilmington, DE), 100 mg/ml bovine serum albumin, 200 nmol/L AP primer, and 200 nmol/L upstream primer 5′-CAGCCATGAGGACCCTC-3′ corresponding to the consensus signal sequence of the human defensin genes. PCR was performed for 30 cycles: 1 minute at 94°C, 1 minute at 55°C, and 2 minutes at 72°C. After amplification, the PCR product was diluted with loading buffer, and 3 μl was loaded onto a 5% nondenaturing single-strand conformation polymorphism (SSCP) gel.[36] After 3 hours of electrophoresis, the gel was dried and exposed to x-ray film. Dried gel pieces corresponding to bands that appeared on the x-ray film were dissected out. DNA was eluted, re-amplified, and purified by glass-milk absorption (Bio101, La Jolla, CA). The purified DNA was then incubated in a standard fill-in reaction with T4 DNA polymerase (Boehringer Mannheim, Indianapolis, IN) and subcloned by ligation to an *Eco*RV linearized blunt-end Bluescript vector (Stratagene, La Jolla, CA). Plasmid DNA was subsequently sequenced by the dideoxy-termination method with Sequenase[36] (United States Biochemicals, Cleveland, OH). Homology searches of all sequences at National Center for Biotechnology Information were performed by BLAST algorithm through the Internet.

RNA Isolation and Reverse Transcription (RT)-PCR

Snap-frozen tissues were pulverized with liquid nitrogen, and total RNA was extracted by the guanidium-thiocyanate-cesium chloride method.[37] RNA was extracted from TriReagent-solubilized epithelial cells following the manufacturer's instructions. RT-PCR was performed using the GeneAmp RNA kit (Perkin Elmer). One microgram of total RNA was diluted to a final volume of 20 μl in a master mix containing 5 mmol/L $MgCl_2$, 1X PCR buffer II, 1 mmol/L dNTP, 1 U of RNAse inhibitor, 2.5 U of reverse transcriptase, and 2.5 μmol/L oligo $d(T)_{16}$ as the primer. The samples were incubated at room temperature for 10 minutes to allow for extension, followed by one cycle of 42°C

for 30 minutes, 99°C for 5 minutes, and 4°C for 5 minutes. A second master mix containing 1 mmol/L $MgCl_2$, 1X PCR buffer II, 0.625 U of Ampli*Taq* Gold DNA polymerase, 0.1 μl of [α-^{32}P]dCTP (3000 Ci/mmol/L), and 0.15 μmol/L of the downstream primer HD5–2R (5′-GAACTGAATCTTGCACTGCTTTGG-3′) and the upstream primer HD5–2L (5′-TGATGAGGCTACAACCCAGAAGC-3′) (Genosys, The Woodlands, TX) was added to 5 μl of sample to a total volume of 25 μl. Amplification of the cDNA was carried out using 35 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. Five microliters of loading buffer was added to 25 μl of the PCR product, and the samples were run on a 5% polyacrylamide (29:1) gel. The 248-p PCR product was confirmed to be HD-5 by direct PCR sequencing on DNA extracted and amplified from the gel pieces that were aligned with the positive band signals on the x-ray film. For normalization, a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer pair yielding a PCR product of 600 bp was also used for an additional PCR run under the same conditions but for 25 cycles only (Continental Laboratory Products, San Diego, CA). The intensity of the HD-5 bands were scored visually as not detectable or positive. PCR conditions for HD-5, GAPDH, and cytokeratin 18 (see below) were optimized before these experiments using 20, 25, 30, and 35 cycles. Radioactively labeled PCR products were then electrophoresed onto a 10% polyacrylamide gel. Subsequently, the gel was dried and exposed to x-ray film. Densitometry was then performed to determine each gel band density, and saturation was determined when there was no increase in intensity with increasing number of cycles. RT-PCR was also performed on the following RNA controls: human small intestine, small intestinal tissue from a HD-5 transgenic mouse (a kind gift of Dr. K. Huttner, Children's Hospital, Boston, MA), and purified preparations of human polymorphonuclear neutrophils (PMNs) and macrophages.

In a second series of experiments, HD-5 expression was semiquantitatively assessed by densitometry in endometrium by performing an additional PCR with a cytokeratin-18-specific primer pair (upstream primer CK18-F 5′-CGAGAAGGAGACCATGCAAAGC-3′ and downstream primer CK18-R 5′-TGGCAATCTGGGCT TGTAGG-3′ yielding a PCR product of 432 bp) under the same conditions as HD-5. Cytokeratin 18 was used as it is exclusively expressed by simple epithelium,[38] and HD-5 expression was calculated as a normalized HD-5: cytokeratin ratio. These data were subjected to logarithmic transformation to normalize the distribution and stabilize the variance of the dependent variable. An orthogonal polynomial contrast test for polynomial trends[39] was performed in coordination with a one-factor analysis of variance (ANOVA).

Immunoperoxidase Staining

Immunohistochemical analysis of tissues was performed using an anti-HD-5 polyclonal antibody produced by immunizing rabbits with recombinant HD-5 linked to ovalbumin as a carrier molecule[40] and a polyclonal antibody recognizing the neutrophil defensins HNP-1, -2, and -3 (HNP(1–3)).[41] Five-micron-thick sections were cut from paraffin blocks and collected onto Superfrost slides (Fisher Scientific, Pittsburgh, PA). Sections were deparaffinized in xylene, rehydrated through a stepwise series alcohols, and rinsed in 0.05 mol/L Tris buffer, pH 7.6. The diluent used in all subsequent procedures was 0.02 mol/L Tris buffer, pH 7.6, containing 1% bovine serum albumin. Sections were incubated for 20 minutes with 10% goat serum to block nonspecific binding sites, followed by an overnight incubation at 4°C with antibodies to HD-5 (1:300 to 1:500) or HNP(1–3) (1:5000). Normal rabbit serum (10%) served as a negative control for primary antibody. Sections of small intestine were used as a positive tissue control. After rinsing in Tris buffer, antibodies were visualized using a rabbit polyclonal alkaline phosphatase/anti-alkaline phosphatase detection system using fast red as a substrate (Biogenex, San Ramon, CA). Tissues were subsequently counterstained with aqueous hemotoxylin and mounted in Accergel (Accurate, Westbury, NY).

Evaluation of Staining

Stained sections were examined by two independent reviewers. The staining patterns for HD-5 in stratified squamous (vagina and ectocervix) and columnar (endocervix, endometrium, and fallopian tube) epithelium were assessed differently. For stratified squamous epithelium, the following scoring system was used: −, epithelium negative throughout section; +, patchy staining in upper layer, negative middle and basal layer; ++, positive staining throughout upper layer, negative middle and basal layer; +++, positive staining throughout upper layer with some intensely stained areas, positive middle layer, negative basal layer; ++++, intense staining throughout epithelium. For columnar epithelium, the number of positively stained epithelial cells was counted. A minimum of 1000 cells was counted in at least five high-power fields. PMN infiltration as represented by staining with the anti-HNP(1–3) antibody was assessed as follows: −, no PMNs; +, scattered positive cells; ++, focal infiltrates in lamina propria; +++, moderate infiltrates throughout lamina propria; ++++, heavy infiltrates throughout lamina propria and epithelium.

Immunofluorescent Staining for Confocal Microscopy

Tissue sections were deparaffinized and rehydrated as previously described. Buffer used throughout the assay was 0.02 mol/L Tris buffer, pH 8.2, containing 1% bovine serum albumin and 20% donkey serum. Sections were blocked for 1 hour with 20% donkey serum before overnight incubation at 4°C with primary antibodies anti-HD5 (1:200) and anti-HNP(1–3) (1:2000), 20% normal donkey serum, or an irrelevant polyclonal antibody). Sections were washed three times, and proteins were visualized with CY-5-conjugated anti-rabbit IgG (Jackson Immunoresearch Laboratory, West Grove, PA). Sections were

subsequently treated with a 1:1000 dilution of the membrane dye RH414 (Molecular Probes, Eugene, OR) for 15 minutes and mounted with Gel-Mount (Fisher Scientific). Tissues were examined with a Zeiss Axiovert 100TV inverted microscope coupled to a MRC-1000 confocal scanning unit equipped with a krypton-argon laser, three photomultiplier detectors, and one transmitted light detector. The immunofluorescence of the CY-5-conjugated probe was collected with a bandpass filter at 680/32 nm while being excited at 647 nm. The membrane dye RH414 was excited at 488 nm, and the emission was collected with a bandpass filter of 605/32 nm. All images were acquired with a Zeiss 63× plan-achromat objective.

Purification of Cationic Peptides from Cervicovaginal Lavages

Cervicovaginal lavages (CVLs) were obtained by irrigation of the vaginal cavity with 10 ml of vinegar and water douche (Massengill, SmithKline Beecham, Pittsburgh, PA). CVLs were collected at 7-day intervals over one 28-day menstrual cycle. Cells were removed by centrifugation, CVLs were neutralized to pH 7.0 with ammonium hydroxide, and cationic peptides were extracted with 200 μl of CM MacroPrep beads (Bio-Rad, Hercules, CA) for 30 minutes at room temperature with constant agitation. The beads were subsequently washed three times with 25 mmol/L ammonium acetate, pH 7.5, containing 0.25% acetic acid, and the peptides were eluted with 1 ml of 5% acetic acid for 20 minutes at 4°C, followed by an additional 1 ml of 5% acetic acid overnight. The resulting supernatants were pooled, lyophilized, and resuspended in 30 μl of 5% acetic acid.

Western Blot Analysis

Cationic peptides isolated from CVLs were analyzed by acid urea polyacrylamide gel electrophoresis (AU-PAGE) followed by Western blot analysis.[34,40] One-third of each sample was used per analysis, and recombinant HD-5 propeptide (8.5 kd) and mature peptide (3.5 kd) produced using the insect cell/baculovirus protein expression system were used as controls (30 to 500 ng).[40] Peptides were transferred from AU gels to Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA) for 10 minutes with 5% acetic acid using a Bio-Rad Transblot apparatus. Blots were fixed for 2 hours with 0.05% glutaraldehyde in Tris-buffered saline (TBS), washed with distilled water, blocked for 30 minutes in 3% gelatin in TBS, and incubated overnight with a 1:2000 dilution of anti-HD-5 polyclonal antibody in TBS with 1% gelatin and 0.01% ethylmercurithiosalicyclic acid.[41] Blots were washed three times for 10 minutes with TBS containing 0.05% Tween 20 (TTBS), incubated in a 1:500 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Amersham Life Sciences, Little Chalfont, UK) for 1 hour, washed in TTBS for 30 minutes, and then developed using the enhanced chemiluminescence detection system (Amersham Life Sciences).

Table 1. Human Defensin-5 mRNA Expression in Female Genital Tract Tissues

	Number of samples	Number positive (%)
Vagina	10	10 (100%)
Ectocervix	14	13 (93%)
Endocervix	15	9 (60%)
Endometrium	28	18 (64%)
Fallopian tube	17	5 (29%)
Fallopian tube, inflamed	4	4 (100%)

RT-PCR was performed for human defensin-5 of vagina, ectocervix, endocervix, endometrium, and fallopian tube from premenopausal women. Massive infiltrates of polymorphonuclear neutrophils were seen in the inflamed fallopian tube specimens.

Results

Identification of HD-5 Transcript in Vaginal Epithelium

The 3′-RACE PCR products generated from RNA obtained from an enriched sample of vaginal epithelial cells were subjected to SSCP analysis to differentiate PCR products with approximately identical molecular weight (data not shown). Multiple mobility shift bands were identified, and subsequent cloning and sequencing of the DNA isolated from these bands showed two different DNA species. The first was a 425-bp DNA fragment that by a homology search corresponded to the human defensin-1 (HNP-1) cDNA, which is expressed by neutrophils, and was assumed to be a contaminant in the epithelial cell preparation.[11] The second species was a 421-bp DNA fragment. A homology search showed that it corresponded to human defensin-5 (HD-5) cDNA, which has previously been identified in human Paneth cells.[21]

Expression of HD-5 mRNA in Reproductive Tract Tissue

To locate the potential sites of HD-5 message expression in the female genital tract, RT-PCR was performed on multiple, normal, epithelium-enriched tissues collected from vagina, ecto- and endocervix, endometrium, and fallopian tube. The results are summarized in Table 1 and illustrated by representative tissues in Figure 1, A and B. In summary, all vaginal and all but one of ectocervical specimens (93%) expressed detectable HD-5 message. Ascending the tract, we also observed that 60% of endocervical specimens and 64% of endometrial samples, but only 29% of the tubal samples, expressed HD-5 message. During the course of tissue collection, four fallopian tube specimens were identified as highly inflamed, as evidenced by the presence of heavy infiltrates of PMNs. All four of these fallopian tubes expressed HD-5 message, with two tissues expressing the highest levels we ever observed in tissue samples (Figure 1A). A purified preparation of PMNs did not express HD-5 message (Figure 1A).

As HD-5 expression in the upper tract was highly variable, we investigated whether endometrial HD-5 mRNA levels correlated with stage of the menstrual cycle. HD-5

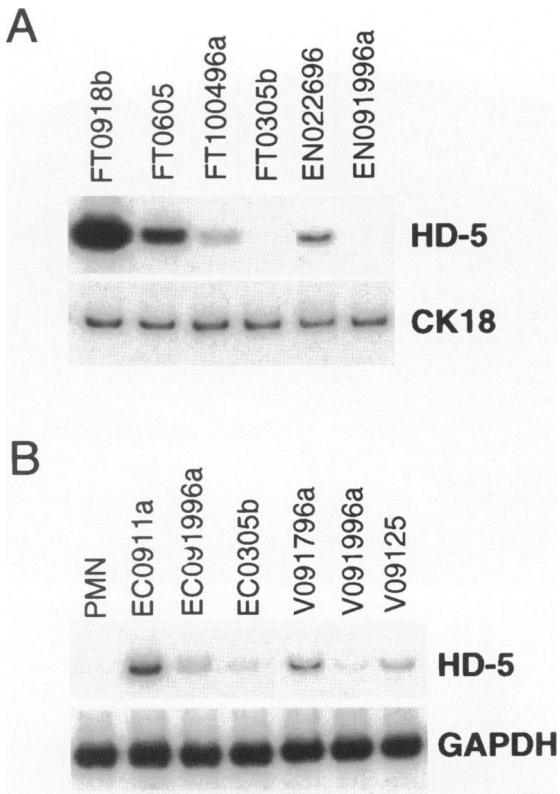

Figure 1. RT-PCR of human defensin-5-specific mRNA from representative female reproductive tract tissues. **A:** Inflamed (FT0918b and FT0605) and normal (FT100496a and FT0305b) fallopian tube and normal endocervix (EN) normalized with cytokeratin 18 (CK). **B:** Normal ectocervical (EC) and vaginal (V) tissue samples and a preparation of purified polymorphonuclear neutrophils (PMNs) normalized with GAPDH. The estimated size of the PCR products was 248 bp for HD-5, 432 for CK-18, and 600 bp for GAPDH.

expression in endometrium was normalized using cytokeratin 18 expression, and samples were categorized as early proliferative (days 1 to 7), mid-late proliferative (days 8 to 14), early secretory (days 15 to 21), and mid-late secretory (days 21 to 28). As shown in Table 2 and Figure 2, HD-5 message expression was low during the early proliferative phase, increased during the mid-late proliferative phase, peaked during the early secretory phase of the cycle, and decreased during the mid-late secretory phase. A statistically significant quadratic

Table 2. Menstrual-Cycle-Dependent Expression of Human Defensin-5 in Endometrium

		Normalized cytokeratin 18: HD-5 ratio	
Stage of menstrual cycle	*n*	Median	Mean ± SE
Early proliferative	7	4.0	11.1 ± 7.3
Mid-late proliferative	5	28.0	32.0 ± 11.9
Early secretory	6	37.0	68.8 ± 32.0
Mid-late secretory	8	6.5	7.4 ± 2.9

RT-PCR was performed for HD-5-specific RNA in menstrual-cycle-dated endometrial tissues. HD-5 expression was normalized using cytokeratin 18. HD-5 message expression in mid-late proliferative and early secretory phases of the menstrual cycle was significantly higher than in early proliferative and mid-late secretory phases ($P = 0.026$ using an orthogonal polynomial contrast test in coordination with a one-factor analysis of variance).

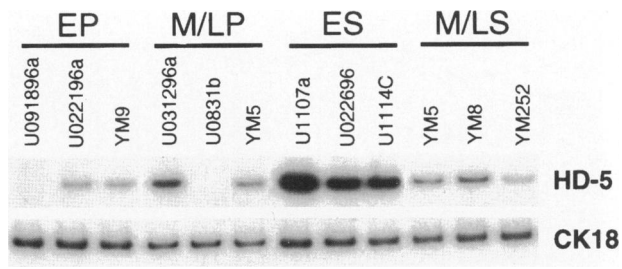

Figure 2. RT-PCR for human defensin-5-specific RNA from endometrial tissue (U) taken at different points in the menstrual cycle and normalized with cytokeratin 18 (CK). Tissues expressing the three median HD-5 values for each cycle stage are illustrated. EP, early proliferative (menstrual); M/L P, mid-late proliferative; ES, early secretory; M/L S, mid-late secretory phase. A statistically significant quadratic contrast indicated that HD-5 mRNA levels in the mid-late proliferative and early secretory groups were higher than early proliferative and mid-late secretory groups ($P = 0.026$).

contrast indicated that HD-5 expression in the mid-late proliferative and early secretory groups were higher than early proliferative and mid-late secretory groups ($P = 0.026$). This pattern of expression was also observed in the smaller group of endocervical specimens collected but did not reach statistical significance.

Immunolocalization of HD-5

To assess the presence and localization of HD-5 protein in the reproductive tract, we used a polyclonal antibody to HD-5[40] in combination with immunoperoxidase labeling.[35] We also included a polyclonal antibody that is specific for the most abundant neutrophil α-defensins, which are human neutrophil defensins-1, 2, and 3 (HNP(1–3)).[41] All vaginal and ectocervical tissues examined expressed HD-5 protein. In these tissues, HD-5 staining was exclusively located to the stratified squamous epithelial cells, and the pattern of staining was similar in both tissues (Figure 3A and Table 3). Positive cells were generally found only in the upper half of the epithelium, with the intensity of cellular staining increasing toward the lumen. Scattered clusters of cells at the luminal surface produced the strongest staining. The ectocervical and vaginal epithelium were negative for HNP-1, but cells that were PMN in their morphology were strongly positive (Figure 3B). Secreted HNP-1 was very often seen in the stroma adjacent to positive cells, and as recently reported elsewhere, the endothelium of some blood vessels was also positively stained.[42]

A survey of endocervix, endometrium, and fallopian tube revealed HD-5-specific staining of the columnar epithelial cells in some, but not all, tissues. In positive samples, immunohistochemical staining revealed that HD-5 was located at the apical surface of between 1 and 14% of ciliated epithelial cells (Figure 3, D and F). In the inflamed fallopian tube specimens, HD-5 protein expression was highly unregulated, with up to 47% of the epithelial cells positive for HD-5 and a greater intensity of staining per cell (Figure 4, C and D). The columnar epithelium of the upper tract was negative for HNP-1, but scattered cells of PMN-like morphology were HNP-1 positive. In the inflamed fallopian tube samples, heavy infil-

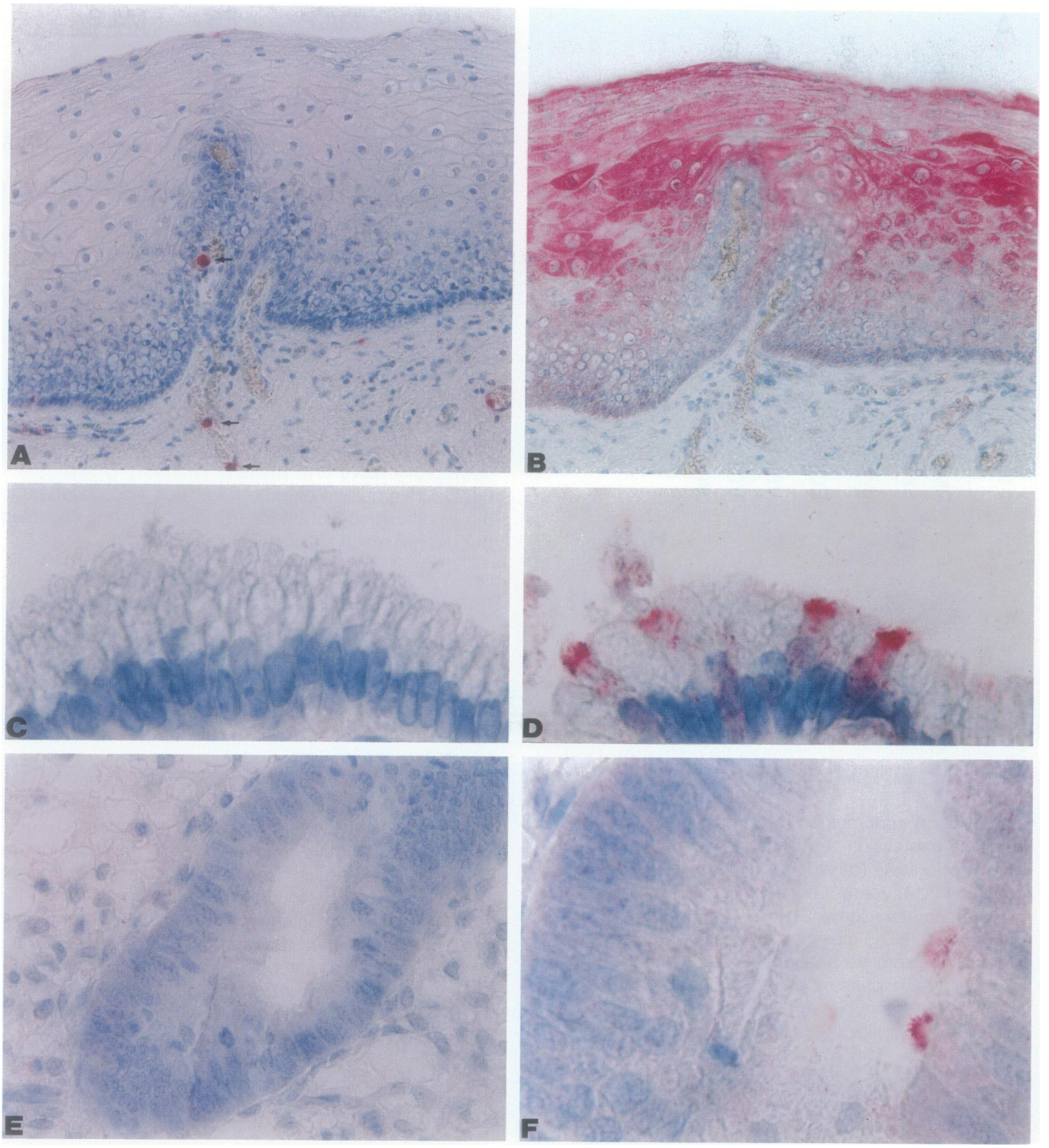

Figure 3. Immunostaining of normal vagina, endocervix, and endometrium with antibodies against human defensin-5 (anti-HD-5) and human neutrophil defensins 1, 2, and 3 (anti-HNP(1–3)). **A** and **B:** Vaginal tissue reacted with polyclonal antibodies against HNP(1–3) (**A**; positive cells marked by **arrows**) and HD-5 (**B**). Magnification, ×20. **C** and **D:** Endocervical tissue reacted with normal rabbit serum (**C**) and anti-HD-5 polyclonal antibody (**D**). Magnification, ×100. **E** and **F:** Mid-proliferative phase endometrium reacted with normal rabbit serum (**E**) and anti-HD-5 polyclonal antibody (**F**). Magnification, ×40 (**E**) and ×100 (**F**).

trates of HNP-1-positive cells and secreted HNP-1 were observed (Figure 4B).

Confocal microscopy provided a greater resolution of HD-5 staining patterns, and use of the membrane dye RH414 allowed us to differentiate between intracellular and extracellularly located HD-5. Staining patterns in stratified squamous epithelia indicated that HD-5 was localized in small granules scattered throughout the cytoplasm of the epithelial cells (Figure 5A). In the columnar epithelial cells of the endocervix, endometrium, and fallopian tubes, HD-5 was located in apically oriented granules and on the outer apical surface of the membrane (Figure 5C). This latter observation suggested that HD-5 was being secreted by columnar epithelial cells.

Table 3. HD-5 Protein Expression in Female Reproductive Tract

Tissue	*n*	HD-5 immunostaining			HNP-1 immunostaining	
		% Protein positive	Median	Range	Median	Range
Vagina	7	100	+++	+ to +++	+	− to ++
Ectocervix	10	100	++	+ to +++	++	− to +++
Endocervix	6	66	1.6	1.1 to 3.4	++	− to +++
Endometrium	14	57	2.3	1.3 to 13.7	+	+ to ++
Fallopian tube	4	50	1.1	1.1 to 5.0	+	+ to ++
Fallopian tube, inflamed	3	100	13.5	2.5 to 46.8	+++	+++ to ++++

For stratified squamous epithelium, the following histological scoring system was used: −, epithelium negative throughout section; +, patchy staining in upper layer, negative middle and basal layer; ++, positive staining throughout upper blayer, negative middle and basal layer; +++, positive staining throughout upper layer with some intensely stained areas, positive middle layer, negative basal layer; ++++, intense staining throughout epithelium. For columnar epithelium, the number of positively stained epithelial cells was counted. A minimum of 1000 cells were counted in at least five high-power fields. PMN infiltration was assessed as follows: −, no PMNs; +, scattered positive cells; ++, focal infiltrates in laminar propria; +++, moderate infiltrates throughout lamina propria; ++++, heavy infiltrates throughout lamina propria and epithelium.

HD-5 secretion

To confirm that HD-5 was secreted into the genital tract lumen, CVLs were collected from one individual with a regular 28-day menstrual cycle at weekly intervals corresponding to early proliferative (menstrual), mid-late proliferative, early secretory, and mid-late secretory phases. The lavage was performed with vinegar-based douche rather than saline as the optimal *in vitro* extraction procedure for defensins requires the presence of acetic acid.[34,40] Cellular material from CVLs was removed by centrifugation, and cationic peptides were subjected to AU-PAGE, which separates molecules primarily by their charge, followed by immunoblotting with an anti-HD-5 antibody. Immunoblots of recombinant peptide and propeptide in the concentration range of 30 to 500 ng were similarly made for reference. As shown in Figure 6, three naturally occurring forms were observed. Two of the forms migrated slower than the recombinant propeptide, and one form migrated between the recombinant propeptide and the more cationic mature peptide. The slowest migrating form was detected only in the day 3 (menstrual) sample. HD-5 secretion appeared to be hormonally dependent, with the lowest concentration detected during menstruation, an increasing concentration during the mid-late proliferative phase, and the highest concentrations occurring in both early and later stages of the secretory phase. Using the immunoreactivity of the recombinant HD-5 forms to estimate HD-5 in CVLs revealed that approximately 1 μg was eluted from the cervicovaginal cavity during the mid/late secretory phase of the menstrual cycle.

Discussion

In this study we located the α-defensin HD-5 in the female genital tract using both RNA and protein detection techniques. We found that HD-5 was invariantly expressed by the stratified squamous epithelium of the normal vagina and ectocervix but was variantly expressed by a proportion of columnar epithelial cells of the normal endocervix, endometrium, and fallopian tube. Secretion of HD-5 was confirmed by detection of the peptide in CVL. Our findings concur with a recent study in which HD-5 transcripts were found in endocervix and endometrium and also in chorionic tissue.[23]

The localization of HD-5 to epithelial cells other than Paneth cells[40] is a novel finding. Paneth cells are highly specialized secretory epithelial cells that are located in the small intestinal crypts of Lieberkühn and contain abundant secretory granules.[43] HD-5 and the mouse Paneth cell homologue cryptdin are located in these granules,[40,44] which also contain a number of antimicrobial molecules, including lysozyme[45] and phospholipase A2 (PLA2).[46] Cryptdin is also associated with the apical brush border of Paneth cells, and diffusely staining material is present in the lumen of the intestine. Isolation of peptides from intestinal fluid has confirmed that cryptdin is secreted into the intestinal lumen.[44]

The immunostaining pattern of cryptdin in the intestine is somewhat analogous to that of HD-5 in the fallopian tubes, endometrium, and endocervix, and we hypothesize that the origin of secreted HD-5 is the epithelium of the upper tract. As only a proportion of columnar epithelial cells are positive for HD-5, it is interesting to speculate that these reproductive tissues also have a specialized epithelial cell population that secrete molecules with antimicrobial activity. Co-localization studies investigating the presence of lysozyme and phospholipase A2 with HD-5 in the upper genital tract are therefore underway.

We detected the presence of natural HD-5 peptides in lavages obtained from irrigation of the vaginal cavity. The peptide present in these CVLs should represent epithelial secretion as cells have been removed by centrifugation. However, contamination of the CVLs with intracellular material of the disrupted epithelial cells cannot be totally excluded. The three forms of HD-5 that were detected in CVLs differed in their electrophoretic mobility in AU-PAGE, which separates peptides mainly according to their charges. All forms migrated slower than the recombinant HD-5 and could reflect amino-terminal or carboxyl-terminal processing of the HD-5 precursor molecule. In addition, secretion of the precursor molecule rather than its active form may occur in the genital tract and has previously been demonstrated for the antimicrobial peptides bactenecin[47] and protegrin.[48] Furthermore, multi-

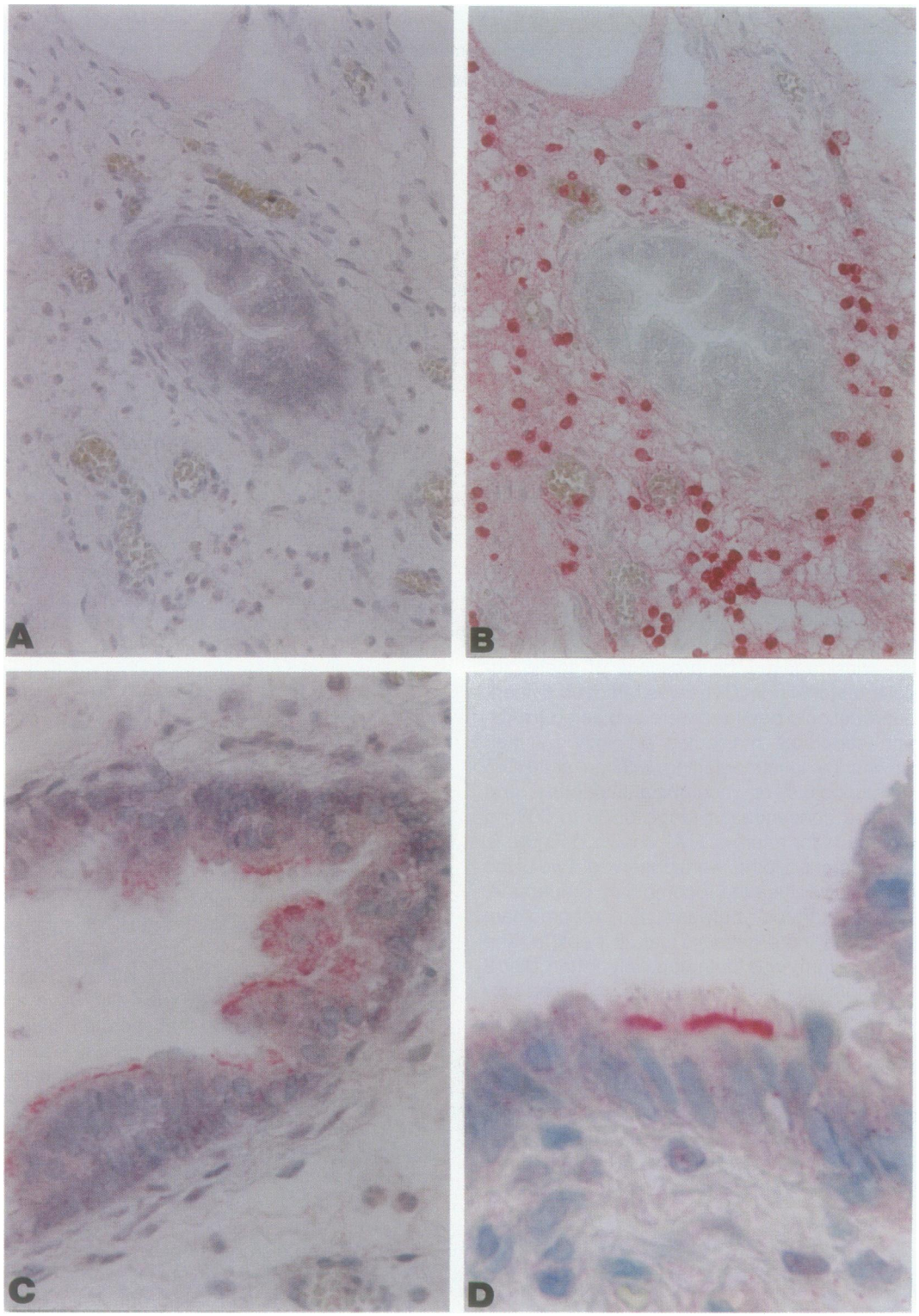

Figure 4. Immunostaining performed on sections of a highly inflamed fallopian tube reacted with normal rabbit serum (**A**), anti-HNP(1–3) polyclonal antibody (**B**), and anti-HD-5 polyclonal antibody (**C** and **D**). Magnification, ×20 (**A** and **B**), ×40 (**C**), and ×100 (**D**).

merization and attachment to other molecules of higher molecular weight or negative charge could also slow down the electrophoretic mobility of natural HD-5. The exclusive presence of the slowest moving form of HD-5 in the menstrual lavage is suggestive of HD-5 binding to a serum protein. Further purification of the HD-5 forms isolated from genital tract secretions is now being undertaken and will eventually allow analysis of its amino terminus, its molecular weight, and its microbicidal activity.

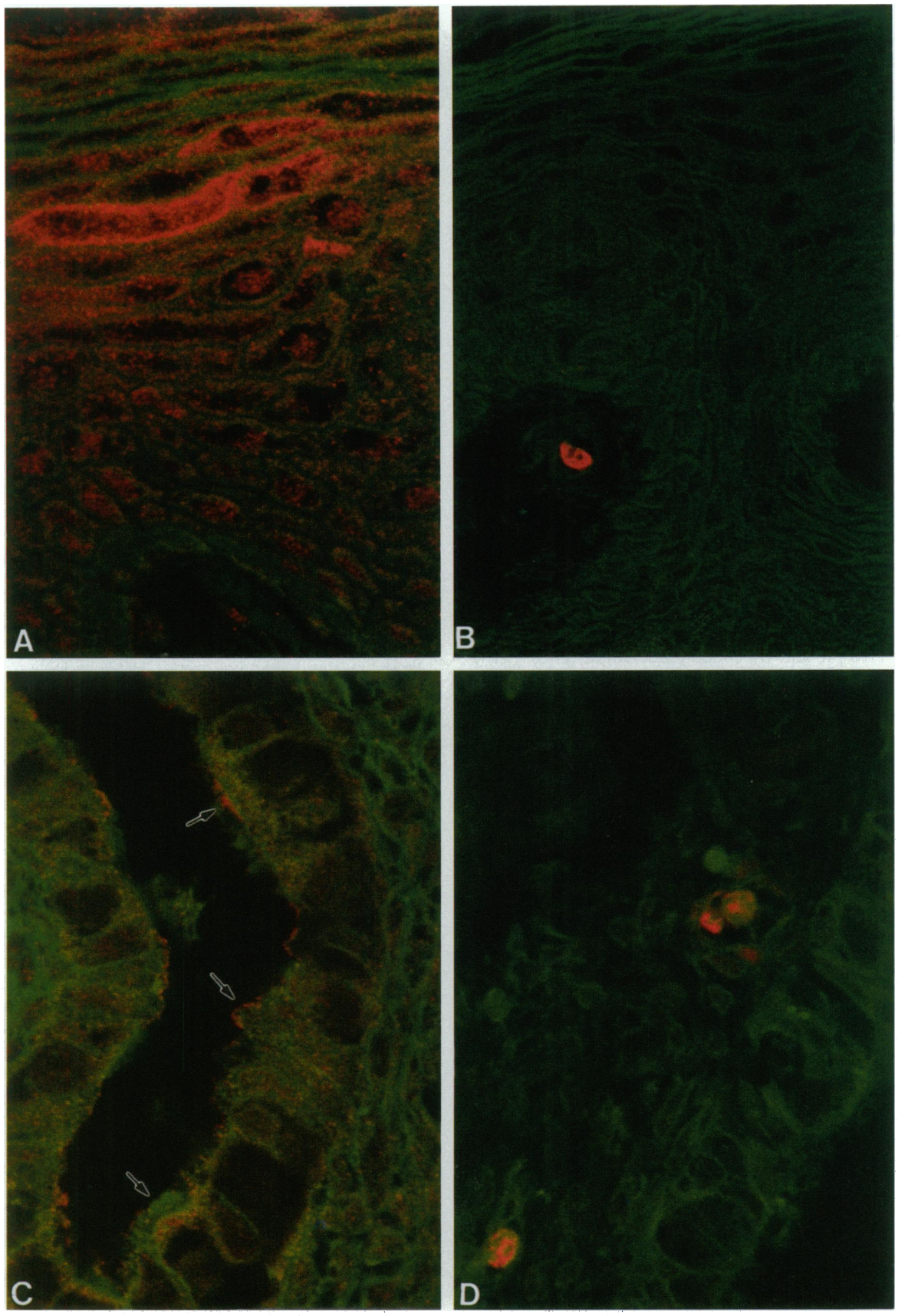

Figure 5. Confocal microscopy performed on representative samples of normal vaginal tissue stained with the HD-5 antibody (**A**) and with the HNP(1–3)-specific antibody (**B**) and fallopian tube stained with the HD-5 antibody (**C**; positive cells are marked with **arrows**) and with the HNP-1-specific antibody (**D**). All tissue sections were also treated with the membrane dye RH414.

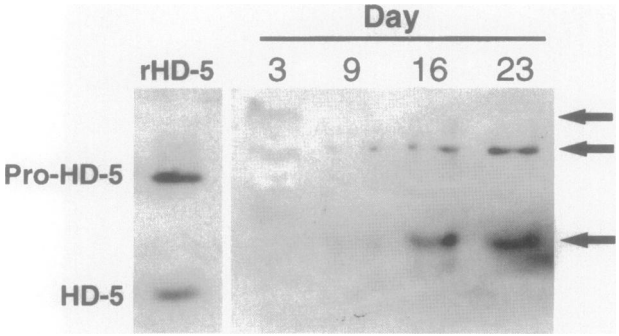

Figure 6. Western Blot analysis of cationic extracts of cervicovaginal lavage subjected to AU-PAGE analysis followed by immunoblotting and probing with anti-HD-5 antibody. rHD-5, recombinant HD-5 propeptide and peptide used for reference (250 ng). HD-5 forms in day 3, 9, 16, and 23 lavages are indicated by **arrows**.

HD-5 expression in adult Paneth cells appears to be constitutive, and up-regulation of HD-5 mRNA has so far been observed only in the Paneth cells of newborns with necrotizing colitis.[49] Consequently, our *in vivo* data demonstrating the up-regulation of HD-5 message and protein in acute and chronically inflamed fallopian tube specimens represent a novel observation. The data we obtained on inflamed tissues is complemented by our recent *in vitro* studies on primary genital epithelial cell cultures, which have shown that HD-5 can be up-regulated by the proinflammatory cytokine tumor necrosis factor (TNF)-α and by lipopolysaccharide (LPS).[50] The inducibility of HD-5 in female genital tract epithelia has a striking resemblance to the pattern of up-regulation seen by some members of the β-defensin family. Thus, *in situ* hybridization studies first indicated that lingual antimicrobial peptide was highly up-regulated in naturally occurring lesions in the lingual epithelium,[26] and additional *in vivo* and *in vitro* studies have since indicated that both lingual antimicrobial peptide and tracheal antimicrobial peptide are up-regulated by LPS and TNF-α and by acute and chronic infection.[51,52] The human β-defensin HBD-2 is also inducible as illustrated by its up-regulation in keratinocyte cultures by TNF-α, bacteria, and fungi,[30] but expression of HBD-1 appears to be constitutive.[33]

The functional role of an epithelial antimicrobial peptide in the innate defense of human respiratory mucosa has recently been investigated. Using a human bronchial xenograft transplant model in *nu/nu* mice, Goldman and co-workers demonstrated that genetically deleting the β-defensin HBD-1 with an antisense nucleotide could completely ablate the antimicrobial activity of human airway fluid secreted by respiratory tract epithelial cells.[32] Furthermore, grafts taken from patients with cystic fibrosis failed to kill bacteria, and this was demonstrated to be a consequence of the high ion content of cystic fibrosis airway fluid, which abrogated the antimicrobial activity of HBD-1.[32] The function of antimicrobial peptides in the female genital tract can only be speculated at this stage. There is no classical mucosal secretory immune system in the uterus,[53] and it is proposed that the endometrium produces powerful innate immune molecules such as antimicrobial peptides to eliminate ascending organisms. The endometrium is particularly vulnerable to infection during the window of ovulation, when the cervical plug is no longer intact and sperm are able to gain access to the uterine cavity. Microorganisms can also ascend into the upper tract at this time, both as free cells and attached to sperm.[54,55] Our results indicate that HD-5 transcripts appear during the mid-late proliferative phase of the menstrual cycle, peak during the early secretory phase, and drop again to baseline during the late secretory phase. HD-5 peptide measured in cervicovaginal fluid was absent in the early proliferative phase and secreted in low levels in the mid-late proliferative phase and in high levels throughout the secretory phase of the cycle. The time difference observed between HD-5 message and protein production may be due to the time period required for translation of message but may also reflect the lag period between production of message, secretion of the peptide, and their detection in lower tract lavages. We propose that defensins are hormonally regulated, presumably by progesterone, with transcription activation occurring several days before ovulation and protein translation not occurring until late secretion. This would ensure the elimination of ascending pathogens during ovulation and maintain the sterility of the upper tract during implantation. The presence of HD-5 would also prevent the inappropriate activation of leukocytes by microorganisms during this critical period. As demonstrated by our data on inflamed fallopian tubes, in addition to cycle-related expression of HD-5 at vulnerable times in the menstrual cycle, HD-5 induction would also occur in the presence of infectious or inflammatory stimuli.

Innate defenses in the lower tract clearly have a different role in comparison with the upper tract. The vagina and cervix are normally colonized by a polymicrobial microflora predominated by lactobacilli species, and vaginal fluid contains approximately 10^9 organisms/ml.[1] Perturbations in the vaginal environment causing the concentration of organisms to increase several log fold results in the condition known as vaginosis, and this increases the susceptibility of an individual to PID.[6] We hypothesize that in a healthy lower genital tract HD-5 is constitutively expressed due to stimulation by commensal bacteria, possibly by LPS. Our immunostaining for HD-5 in stratified squamous epithelium does not indicate evidence of secretion, and we thus predict that HD-5 is not secreted to allow commensal survival but that, if bacterial invasion of the epithelium occurs, HD-5 expression is up-regulated and can function intracellularly.

In summary, our findings have important ramifications for the prevention or treatment of sexually transmitted infections. Studies on the classical neutrophil defensins indicate they have a wide, but variable, spectrum of antimicrobial activity. We hypothesize that unique peptides are produced in the mucosa as their spectrum is biased toward mucosal pathogens. Elucidation of the factors that regulate antibiotic peptides may allow us to manipulate their expression, thus enhancing the antimicrobial potential of the epithelial cell barrier and preventing establishment of infection.

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