Purification of antimicrobial factors from human cervical mucus

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BACKGROUND: The aim of this study was to separate bactericidal proteins from healthy female cervical mucus. METHODS: Cervical mucus was collected and dissolved in 1% acetic acid. The antimicrobial activity of acid-soluble extracts was detected by gel overlay assay against Escherichia coli ATCC 43827. The extracts showed considerable amount of antibacterial activity with a clearly visible band. The bactericidal band was purified by reverse-phase high performance liquid chromatography and the antibacterial activity of the eluate was examined using radial diffusion assay. RESULTS: Two antimicrobial proteins were purified and were further characterized by Tricine sodium dodecyl sulphate-polyacrylamide gel electrophoresis, N-terminal sequencing and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. The proteins were identified as high-mobility group nucleosomal-binding domain 2 (HMG N2) and secretory leukocyte peptidase inhibitor (SLPI). SLPI is an antimicrobial peptide already known in the cervical mucus while HMG N2 in the cervical mucus had not been previously reported. The expression of HMG N2 mRNA was detected in Hela cells and cervical epithelial cells by RT-PCR. Slit hybridization showed abundant amounts of the HMG N2 protein in the cervical mucus. CONCLUSIONS: These results suggest that the expression of HMG N2 and SLPI in the healthy female cervical mucus may be relevant to their immune surveillance and defense against potential pathogens in human reproductive system.

Keywords: innate immune; antimicrobial protein; cervical mucus; high-mobility group nucleosomal-binding domain 2; secretory leukocyte peptidase inhibitor

Introduction

In recent years, antimicrobial peptides and proteins, with the capacity to kill a broad spectrum of microorganisms, have become recognized as key mediators of the innate immunity in many organisms (Ganz, 2005; Silphaduang et al., 2006). Successful human implantation and pregnancy are reliant on the prevention of genital tract infections that can compromise both fertility and the fetus itself. The molecules of the innate immune system, present at mucosal surfaces, are likely to be crucial in the limitation of such infections (King et al., 2002). The female reproductive tract is an important site of antibacterial peptide production. Several antimicrobial peptides have been detected in the reproductive tissues including lysozyme (Eggert-Kruse et al., 2000), human beta defensin (HBD) 1–4 (King et al., 2002; King et al., 2003), human defensin (HD) 5 (Svinarich et al., 1997) and secretory leukocyte peptidase inhibitor (SLPI) (King et al., 2000; King et al., 2002). Our group has also found that HBD 1 and HBD 2 are located in female genital tract and pregnancy-related tissues (Feng et al., 2003). The presence of these molecules suggests a role in the protection of the reproductive tract from infections.

Cervical mucus is a viscous, gel-like fluid located between microbe-rich vagina and the normally sterile uterine cavity, which suggests a host defense function other than a physical barrier preventing ascending infections (Hein et al., 2001). The cervical mucus is well known as a multifactorially determined filtering system, but not much is known about its antimicrobial activity (Eggert-Kruse et al., 2000). Even though antibacterial activity of cervical mucus had been found previously, none of the antimicrobial peptides, except SLPI (Moriyama et al., 1999) and lysozyme (Eggert-Kruse et al., 2000), had been purified from the cervical mucus. The aims of the present investigation were to identify novel antibacterial molecules and characterize these molecules as potentially important factors in the innate immunity of human
reproductive tract. Our results showed that cervical mucus exhibited antibacterial activity in vitro and two antimicrobial factors (including one previously in reported factor) were separated from it.

Materials and Methods

Collection of cervical mucus

The Regional Research Ethics Committee approved the project and consent was obtained from each volunteer. The women (n = 8) were healthy, with a median age of 26 (range 24–35) years. The parity was 1, and the specimens were taken at least one year after delivery. All the women had regular menstrual cycles (27–31 days). None of them had taken any antibiotics for at least 4 weeks or hormones for 3 months prior to biopsy collection. At least 5 days before material collection, the volunteers refrained from sexual intercourse. They presented with a normal-appearance cervix and without pathological cervical discharge or symptoms of genital tract infection. All the samples were obtained at 3–4 days after menstruation by aspiration with a 1 ml syringe from the cervix.

The samples were dissolved in 1% acetic acid, homogenized and centrifuged at 12000g for 30 min at 4°C. The supernatant was collected and dialysed against water overnight at 4°C. The samples were then lyophilized and re-dissolved with 0.01% acetic acid. Protein concentration was measured by bicinchoninic acid protein assay (Pierce Biotechnology Inc, USA). All samples were stored at −80°C for future use.

Gel overlay antibacterial assay

Gel overlay assay was performed according to the protocol of Lehrer et al. (1991). Briefly, Escherichia coli ATCC 43827 cells were grown overnight in Luria–Bertani broth and centrifuged at 12000g for 10 min at 4°C. The pellet was washed twice using 20 mM phosphate-buffered saline (PBS) (pH 7.2). The bacterial concentration was adjusted to 1×10^8 CFU/ml by using a colorimeter (Bio-rad Inc, USA). Then the bacteria were mixed with underlay gel solution kept molten at 43°C to have the final concentration of bacteria at 1×10^5 CFU/ml. The underlay gel was composed of 1% agar, 0.3% soybean extract and 100 mM phosphate buffer (pH 6.8). The mixture was immediately poured into an integrid Petri dish (Fisher scientific, USA) to form a uniform layer of 1 mm deep.

Crude extracts (30 μg) of cervical mucus were subjected to non-denaturing acid–urea polyacrylamide gel electrophoresis (AU-PAGE) in duplicate. After electrophoresis, the gel was cut into two identical halves. One half was washed by rinsing in 0.01 M PB (pH 7.2) for 10 min to decrease the acetic acid and urea content. Then the PAGE gel was placed on the top of the underlay gel. The dish was incubated for 3 h at 37°C to allow the peptides to diffuse into the underlay gel from the PAGE gel. The PAGE gel was removed and replaced by an overlay gel containing 1% agar and 6% soybean extract. The chimeric gel was incubated overnight at 37°C, to enable the surviving bacteria to form microcolonies. Clear colony-free regions were seen where the growth of bacteria was suppressed by protein bands with antibacterial activity. The second half of the gel, an exact replica of the first half, was stored in 0.01% acetic acid in MilliQ water, and was used for the excision of antimicrobial zones.

Purification of the antimicrobial proteins

Peptides were excised from the AU-PAGE replica and purified. The purification included preparative AU-PAGE elution and reverse-phase high performance liquid chromatography (RP-HPLC). The preparative AU-PAGE elution was referenced from the protocol of Harwig et al. (1993). The eluates of preparative AU-PAGE were lyophilized, reconstituted in 0.01% acetic acid and then injected into RP-HPLC with a 4.6×250 mm Vydac C18 column (Agilent Inc, USA). Bound materials were eluted with a linear gradient of acetonitrile (ACN) in acidified water [0.1% trifluoroacetic acid (TFA) in water to 60% ACN with 0.1% TFA] for 60 min at a flow rate of 1.0 ml per minute. The elution profile was monitored at 214 nm. All fractions were collected, lyophilized, reconstituted with 0.01% acetic acid and stored at −80°C for further tests of their antimicrobial activities.

Radial diffusion antimicrobial assay

The underlay gel containing bacteria was prepared exactly as described above for the gel overlay assay. A series of wells, each 3 mm in diameter, were punched into the solidified underlay gel. Around 5 μl of samples (1 μg) or positive control (lysozyme, 10 μg) was added into designated wells. The dish was incubated at 37°C for 3 h to allow the peptide to diffuse into the underlay gel from the wells. A molten overlay gel was poured, and the dish was incubated overnight at 37°C until zones of inhibition were visible. The clear zones around the holes indicating no growth of bacteria were seen when the samples contained antibacterial effectors.

Electrophoresis and electrotransfer to membrane

Tricine sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Tricine SDS-PAGE) for low-molecular weight proteins was performed as described by Schagger et al. (1987). The SDS gel had a separating gel of 16.5% acrylamide with a 10% acrylamide spacer gel and 4% stacking gel. The gel was subjected to electrophoresis for 3 h at 80 V and stained by silver staining. For the samples used in N-terminal sequencing or mass spectrometry analysis, the peptides were electro-transferred from gel to membrane before staining. Electrottransfer was performed at 20 V for 40 min. ProBlott polyvinylidyene difluoride (PVDF) membranes (0.45 μm, Invitrogen Inc, USA) stained with Coomassie Brilliant Blue R-250 (Sigma, USA) were used for sequence analysis while nitrocellulose membranes (0.22 μm, Invitrogen Inc) stained with Amido Black were used for mass spectrometry.

N-terminal sequencing

The amino acid sequencing was done at Peking University, Beijing, China. N-terminal amino acid sequencing of the antimicrobial factor was performed by sequential Edman degradation method using Procise 477 automatic sequence analysis system (Applied Biosystems, USA) according to standard protocols of manufacturer.

Mass spectrometry

Protein identification by mass spectrometry analysis was performed at Chengdu Branch of Chinese Academy of Sciences, Chengdu, China. The molecular weight of the isolated antimicrobial factor was determined by a mass spectrometric method using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS) instrument (Bruker Daltonics, Germany).

Primary epithelial cell culture

Cervical tissues (n = 5) were obtained from women undergoing total hysterectomy because of hysteromyoma or endometriosis. Consent was obtained from each patient and the investigation was approved by the Regional Research Ethics Committee. Cervical tissues were checked by pathologic examination. Samples without pathologic changes were used in following procedure. Cervical tissues were digested by 0.1% protease and 0.1% DNAase at 4°C for 36 h. The supernatant was collected and then centrifuged at 1000g for 10 min. The pellet was washed three times with PBS, for 5 min each time.
The primary cervical epithelial cells were cultured according to the protocol of Gorodeski et al. (1994) with some modification. Briefly, the epithelial cells were cultured in 153 culture medium (Sigma) supplemented with Insulin (5 g/ml, Sigma), hydrocortisone (0.5 g/ml, Sigma), transferrin (5 g/ml, Sigma), epidermal growth factor (10 ng/ml, Sigma), epinephrine (7.5 g/ml, Sigma), bovine pituitary extracts (20 ng/ml, Sigma), penicillin (50 µg/ml, Sigma) and streptomycin (50 µg/ml, Sigma). There is evidence that these factors are likely to be involved in modulation of cell proliferation, differentiation and maturation (Stanley et al., 1984; Chopra et al., 1991; Gorodeski et al., 1994; Myohanen et al., 2001). Cells were grown to near confluence (7–9 days) in a 6-well plate.

**Cell lines**

Hela cells (Shanghai Branch of Chinese Academy of Sciences, Shanghai) were grown in RPMI 1640 (Gibcol BRL, USA) supplemented with 10% fetal calf serum (Gibcol BRL), penicillin (50 µg/ml, Sigma), streptomycin (50 µg/ml, Sigma) and epidermal growth factor (50 µg/ml, Sigma). There is evidence that these factors are likely to be involved in modulation of cell proliferation, differentiation and maturation (Stanley et al., 1984; Chopra et al., 1991; Gorodeski et al., 1994; Myohanen et al., 2001). Cells were grown to near confluence (7–9 days) in a 6-well plate.

**Nucleic acid extraction and RT-PCR analyses**

Total RNA was extracted with Trizol reagent (Invitrogen Inc) from the cervical epithelial cells and Hela cells. RNA samples were then treated with RNase-free DNase I (Promega, USA) to remove cellular DNA. The Superscript II (Invitrogen Inc) was utilized to synthesize cDNA. A PCR measurement and agarose gels electrophoresis. The Superscript II (Invitrogen Inc) was utilized to synthesize cDNA. A PCR reaction was performed with sense primer (5’AACGGATCC TTTGACCCTGCTG) and antisense primer (5’CCAGGATCCG CACACACTAG). A PCR reaction mixture was made to contain cDNA (3 µl), rTaq DNA polymerase (0.025 IU/ml; Takara, Japan), MgCl₂ (2 mM), dNTPs (all at 200 mM) and specific sense and anti-sense primers (300 nM; Takara). A no template control (containing water) was included. The PCR reactions were run on Eppendorf Mastercycler 5331 (Eppendorf, German) and the thermocycling conditions were as follows: initial denaturation for 5 min at 95°C; followed by 30 cycles of 95°C for 45 s, 52°C for 30 s and 72°C for 30 s, and then final extension for 7 min at 72°C. All cDNA synthesis/RT-PCR experiments were performed at least three times. The products were analysed by electrophoresis through 2% agarose gel and DNA sequencing.

**Slit hybridization of human cervical mucus**

The expression of recombinant high-mobility group nucleosomal-binding domain 2 (HMG N2) protein and preparation of rabbit anti-HMG N2 polyclone antibody were performed by our laboratory as described elsewhere (Xiong et al., 2005). Slit hybridization was carried out using the Hoefer Dot Blot system (GE, USA) following the protocol provided by the manufacture. Briefly, 5 µl of extract proteins from human cervical mucus (15 µg) were dotted on PVDF membrane and dried before proceeding to the blocking step. Subsequently, the membrane was blocked with blocking buffer (1% nonfat dry milk, 20 mM Tris–HCl, 500 mM NaCl, 0.1% Tween-20, pH 7.5) for 2 h with gentle shaking at room temperature. After incubation the membrane was washed with washing buffer (20 mM Tris–HCl, 500 mM NaCl, 0.1% Tween-20, pH 7.5) for 10 min with gentle agitation. The membrane was then incubated overnight at 4°C with rabbit anti-HMG N2 antibody diluted to 1:1000 in the blocking buffer. After overnight incubation the membrane was rinsed twice with washing buffer before being incubated with goat anti-rabbit IgG-horseradish peroxidase second antibody (Dako, Denmark), which was diluted to 1:5000 in blocking buffer, at room temperature for 1 h. To remove the excess antibody, the membrane was washed twice with washing buffer. Finally, color signals of the membrane was obtained following incubation with the 3, 3-diaminobenzidine (Dako) for 10 min, and washed with distilled water to stop the reaction. Recombinant HMG N2 protein was used as positive control and bovine serum albumin (BSA), as negative control.

**Results**

**Tissue collection**

We found that the cervical mucus was a vicious hydrogel weighing between 0.15 and 0.30 g. Examined with paper strips, the median pH of the cervical mucus was 7.2, with a range of 6.4–8.0. The cervical index was determined (Insler et al., 1972) and the score of cervical mucus was no greater than 2 in all donors. The mucus samples contained no blood contamination. The number of leukocytes was <1 (mean of 10 visual fields) per high power field (×400).

**Antibacterial activity of human cervical mucus**

The antibacterial activity of acid-soluble proteins from human cervical mucus was tested by the gel overlay assay (Fig. 1). Lysozyme, a known antibacterial peptide, was used as positive control. The electrophoresed antimicrobial proteins were separated on a polyacrylamide gel and the molecules were transferred to a bacterial-inoculated agarose gel (the underlay gel described above) by blotting. After overnight incubation, bacteria-free regions were clearly visible. Because proteins with similar ionic charge cannot be separated by AU-PAGE, the band containing antibacterial activity maybe includes several proteins. Therefore the band was cut off from the PAGE gel for further separation and purification.

**Purification of antimicrobial proteins**

The active band was separated by RP-HPLC into >10 fractions (Fig. 2A). Radial diffusion assay was used to assess the...
microbicidal activity of eluates. Two peaks, fraction 21 (eluted at 21 min) and fraction 26 (eluted at 26 min), presented high levels of antimicrobial activity against *E. coli* ATCC 43827 (Fig. 2B).

**Electrophoresis of selected chromatography fractions**

The fractions 21 and 26 shown in Fig. 2B were analysed by Tricine SDS-PAGE electrophoresis and stained by silver staining (Fig. 2C). Fractions 21 and 26 displayed only one protein band each, at ~14 and 12 kDa, respectively (Fig. 2C).

**Identification of proteins**

The first 10 amino acid residues of the N-terminal sequence of fraction 21 were P-K-R-K-A-E-G-D-A-K, which was exactly corresponding to the N-terminal sequence of the human HMG N2 through the BLAST program assay (http://www.ncbi.nlm.nih.gov/BLAST/). Molecular mass of fraction 21 was determined using MALDI-TOF-MS. The molecular mass of fraction 21 was 9263.62 Da (Fig. 3), in a good agreement with the molecular mass of HMG N2. It suggested that fraction 21 should be as entire molecules of HMG N2, an antibacterial effector not previously detected in reproductive tract.

The molecular mass of fraction 21 determined by MALDI-TOF-MS was lower than the relative mass for fraction 21 indicated by mobility on Tricine SDS-PAGE (Fig. 2C), which was 14 kDa. It may be explained by the aberrant behavior of HMG protein in these conditions as reported by Einck et al. (1985). The first 10 amino acid residues of the N-terminal sequence of fraction 26 were S-G-K-S-F-K-A-G-V-C, which was identical to the N-terminal sequence of SLPI. The calculated molecular weight of SLPI is 11.7 kDa, in exact agreement with the molecular mass observed in the Tricine SDS-PAGE. It has been reported that SLPI is a constitutively expressed natural antibiotics in female reproductive tract (King et al., 2000, 2002). Therefore fraction 26 was identified as SLPI.

**HMG N2 expression in cervical epithelial cells**

Employing RT-PCR, we revealed the presence of HMG N2 mRNA in the cervical epithelial cells and Hela cells. Agarose gel electrophoresis of PCR products showed that there was a specific band at about 270 bp (Fig. 4), in accordance with our expectation. The results of DNA sequencing confirmed that the fragment was 270 bp in length, and identical to HMG N2.

**Slit hybridization of human cervical mucus**

A slit hybridization assay was performed to prove the hypothesis that HMG N2 is present in human cervical mucus. Immunostaining was observed in the cervical mucus and positive control, whereas no staining was visible in negative control. The results revealed the presence of the HMG N2 protein in the cervical mucus of healthy women (Fig. 5).

**Discussion**

The antimicrobial activity of cervical mucus had been noted, but its content of antimicrobial peptides has not been systematically analysed. This investigation focused specifically on the separation and identification of new antibacterial effectors...
from healthy female cervical mucus. In the present study, two antibacterial peptides, HMG N2 and SLPI were identified.

The HMG protein family consists of six proteins and each of these proteins seems to have a distinct type of function in the nucleus (Bustin, 1999). However, it is well known that peptides of the HMG protein family have additional functions. For example, the HMG box chromosomal protein 1 (HMG B1) is widely known as a nuclear DNA-binding protein. Now, there is growing body of evidence demonstrating that HMG B1 possesses potent bactericidal activity (Zetterstrom et al., 2006). HMG N2 gene is located in chromosome 1p36.1, and it contains six exons, with an extremely high GC content and an ‘Hpa II tiny fragment’ island. Until now, the biological role of HMG N2 has not been defined fully. A variety of experiments have shown that HMG N2 is associated with chromatin subunits (Hock et al., 1998). Furthermore, the abnormal gene or protein expression of HMG N2 is related to some diseases such as neoplasms (Spieker et al., 2000) and autoimmune diseases (Ayer et al., 1994). The significance of HMG N2 in the host innate immune against infections was reported by our laboratory (Feng et al., 2005). A previous study showed that HMG N2 was an antimicrobial molecule with potent activity against E. coli, Pseudomonas aeruginosa and Candida albicans. An amphipathic α-helical domain was found in the HMG N2 protein, which was proved to be essential for its antimicrobial activity. The present study demonstrated HMG N2 located in female cervical mucus, which was further confirmed by slit hybridization. To our knowledge, this is the first report describing detection of HMG N2 presented in human cervical mucus. The mRNA of HMG N2 was detected in Hela cells and primary cervical epithelial cells using RT-PCR, which indicated that HMG N2 was expressed in cervical epithelial cell. Thus, HMG N2 could be regarded as antibacterial effector released by the cervical epithelial cells to combat invading bacteria in reproductive system.

SLPI has been previously detected in female reproductive system, including the cervix (Helming et al., 1995), endometrium (King et al., 2000, 2002), first trimester decidua (King et al., 2000), term decidua (Hiemstra et al., 1996) and amnion fluid (Denison et al., 1999). SLPI was thought to function mainly as a serine protease inhibitor. Now, SLPI has been found to have antibacterial, antiviral and antifungal effects (Zhang et al., 2001). Our observation of SLPI in cervical mucus confirmed the previous reports. It should be noted that SLPI still had the ability to protect antimicrobial peptides from excessive proteolysis and inactivation by inflammatory proteases (Tomee et al., 1998).

The identification of antimicrobial factors in cervical mucus was based on the method of Bourgeon et al. (2004). The advantage of this method was that it involved the direct identification of molecules responsible for the observed antimicrobial effects. It is of great potential value for the identification of novel antimicrobial effectors in immunoprivileged biological fluids and tissues. Several antibacterial molecules have been identified using this method from different tissues including the male and female reproductive tract (Bourgeon et al., 2004; Mak et al., 2004; Silphaduang et al., 2006). As cervical mucus is a viscous gel-like fluid, solubilization is necessary to ensure a homogeneous mixture. Sonification was proved to be an efficient method for dispersion of cervical mucus (Eggert-Kruse et al., 2000). It was used in all samples. Enzymatic treatment was not used because of its proteolytic activity. The antibacterial molecules studied to date are all cationic peptides with a high isoelectric point (Reddy et al., 2004), therefore, more soluble in a mild acid solution. Acid extraction of tissue was performed to enhance the solubility of the antibacterial proteins. In fact, the antibacterial activity of cervical mucus extracted by acid solution was more potent than when extracted by water solution. Similar results were obtained by King et al. (2000).

Moreover, no other antimicrobial peptides, such as defensins and lysozyme, were detected in the cervical mucus by using our experimental method. We postulated that the healthy female used in this study did not constitutionally produce such molecules, or these factors were expressed at a low level that our method could not detect it. It has been shown that defensins are inducible antimicrobial peptides in epithelial cells of endometrial (King et al., 2002) and other multiple organs (Bals et al., 1999; Milner et al., 2003). The possible reason that lysozyme was not detected in our study might be that the approach we used was different from Eggert-Kruse’s (2000). A previous paper reported that HNP1-3, β-defensin, lactoferrin and lysozyme are present in cervical mucus plugs from pregnant females (Hein et al., 2001). It is considered that the antibacterial activity is augmented in pregnancy because of the different endocrine status. For example, the concentrations of lysozyme in cervical mucus plugs are about twice as high as in cervical mucus. Therefore, healthy females may have to rely on constitutively expressed immune surveillance factors, such as HMG N2 and SLPI, for a basal level of immune defense, while others are inducible allowing maximal antimicrobial activity during infection or pregnancy.

Ascending infections are major cause of infertility and adverse pregnancy outcome. It is reported that up to 20% of preterm births are associated with uterine infections (Romero et al., 1989). The main role for HMG N2 and SLPI in cervical mucus is likely to be in the prevention of ascending infections. Even though the vagina is polymicrobially colonized and is known to be ecological niches, with a specific transient and resident flora, the uterine cavity is usually sterile (Eggert-Kruse et al., 2000). One explanation for this might be the presence of local antibacterial factors in the cervical mucus. HMG N2 and
SLPI were shown to have antimicrobial activity, hence they may constitute cervix surveillance system designed to protect the uterus from infections.

Our study only identified the antimicrobial molecules of cervical mucus from healthy females. The contents of cervical mucus are influenced by several variables such as endocrine status, sexual intercourse and infections. More samples from healthy and infectious donors with different endocrine status’ and different menstruation stages should be collected for further investigation.

In conclusion, we find that female cervical mucus harbors components with bactericidal activity. These components have been identified as HMG N2 and SLPI. The presence of HMG N2 and SLPI in healthy female cervical mucus may be relevant to their immune surveillance and defense against potential pathogens in the human reproductive system.

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