Large Scale Identification of Proteins, Mucins, and Their O-Glycosylation in the Endocervical Mucus during the Menstrual Cycle*

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The mucus filling the human cervical opening blocks the entry to the uterus, but this has to be relative and allow for the sperm to penetrate at ovulation. We studied this mucus, its content of proteins and mucins, and the mucin O-glycosylation in cervical secretions before, during, and after ovulation. Cervical mucosal secretions from 12 subjects were collected, reduced-alkylated, separated with polyacrylamide or agarose/polyacrylamide gel electrophoresis, and stained with silver, Alcian blue, or Coomassie Blue stain. Protein and mucin bands from before and during ovulation were digested and subsequently analyzed by nano-LC-FT-ICR MS and MS/MS. We identified 194 proteins after searches against the NCBI non-redundant protein database and an in-house mucin database. Three gel-forming (MUC5B, MUC5AC, and MUC6) and two transmembrane mucins (MUC16 and MUC1) were identified. For the analysis of mucin O-glycosylation, separated mucins from six individuals were blotted to PVDF membranes, and the O-glycans were released by reductive β-elimination and analyzed with capillary HPLC-MS and -MS/MS. At least 50 neutral, sialic acid-, and sulfate-containing oligosaccharides were found. An increase of GlcNAc-6GalNAcol Core 2 structures and a relative decrease of NeuAc residues are typical for ovulation, and NeuAc-3Gal- epitopes are typical for the non-ovulatory phases. The cervical mucus at ovulation is thus characterized by a relative increase in neutral fucosylated oligosaccharides. This comprehensive characterization of the mucus during the menstrual cycle suggests mucin glycosylation as the major alteration at ovulation, but the relation to the altered physicochemical properties and sperm penetrability is still not understood. Molecular & Cellular Proteomics 6:708–716, 2007.

The cervix connects the vagina with the uterus and is an “open” entry into the female endometrial and abdominal cavities. The cervical mucus fills the opening of the cervix and acts as an important protective barrier preventing pathogens from ascending into the uterus. Secreted into the vagina, the mucus is also important as it traps microorganisms and flushes these out of the vagina to protect both the uterus and the vaginal epithelium. However, the mucus-blocked entry into the uterus cavity has to be relative to allow the sperm to enter at ovulation. This is reflected in the cervical mucosal secretions undergoing cyclical changes in its physicochemical properties as a response to hormonal changes. During ovulation, the mucus volume increases 10–20-fold and becomes less viscous. In fact, altered mucus properties have been shown to be a very good predictor of the fertile window (1). The importance of the cervical mucus is also illustrated by the observation that abnormalities of the cervical secretions are responsible for infertility in about 5–10% of infertile women (2).

Despite its importance, there is still limited biochemical understanding of the composition of the cervical mucus and how this is altered during ovulation. Most of the cervical mucus is water (95–99%), but it also contains a complex mixture of organic components, inorganic ions, enzymes, bactericidal proteins, plasma proteins, and especially mucins. Mucins are large polymeric molecules that contribute to the viscoelastic gel properties of mucus, and they are therefore important to include in a proteome study. However, mucins are complicated to characterize due to their large size, polymeric nature, and heterogeneous glycosylation. The mucin multimers are linked together by disulfide bonds and built from protein monomers of more than 500 kDa. An extensive glycosylation, exceeding 80% carbohydrates, increases the masses to over 2 MDa. The major gel-forming mucin transcript of the endocervical epithelium is MUC5B (3). The amount of MUC5B mucin in human cervical mucus varies during the menstrual cycle and peaks at ovulation, suggesting that this larger mucin species may be an important factor affecting sperm transit to the uterus (4). Studies based on Northern blot and in situ hybridization have further suggested that other mucin genes are expressed by the endocervical epithelium (5).

All mucins contain large mucin domains, also called PTS domains, that are rich in the amino acids serine, threonine,
and proline and provide a high number of attachment sites for O-linked carbohydrates (5, 6). The specific rheological and hydrodynamic properties of mucins can be ascribed to their extensive O-glycosylation. The carbohydrate chains of gel-forming mucins occur as a heterogeneous population of neutral, sialylated, and sulfated oligosaccharides of two and more sugar residues (7). Altered mucin glycosylation during ovulation could accompany altered biochemical properties, but histochemical studies on cervical mucin glycosylation during the menstrual cycle have produced conflicting results regarding such cyclic changes (8–10). In the present study, we performed a more complete analysis of the glycosylation during the menstrual cycle by the use of mass spectrometry (HPLC-MS/MS) aiming to both characterize and obtain semi-quantitative information on cervical mucin glycosylation. Our approach was based on a simple and fast methodology, consuming small amounts of starting material.

Proteome analysis of human cervical proteins including the characterization of mucin components and the mucin glycosylation during the menstrual cycle is thus important for a better understanding of the complicated processes that protect the reproductive tract. However, identification of mucins with proteomics is difficult due to their large size and the presence of the dense O-glycosylation in the mucin domains, blocking the use of these parts of the molecule for protein identification. In addition, several mucins are not completely sequenced and correctly assembled in the public databases, something that has hampered the identification of these proteins due to low hit scores. We addressed and circumvented these problems by compiling a mucin database and by the use of high precision identification of the mucin peptides by ICR-FT mass spectrometry.

To perform as complete a proteome and mucin analysis as possible of the human cervical mucus during the menstrual cycle, the mucus was gently isolated from healthy donors. Many proteins were identified reflecting their origin from the specific secretion, containing exfoliated cells as well as extracellular fluids and blood. No consistent alterations in the protein composition could be detected over the menstruation cycle. The MUC5B mucin was a major component, and its O-glycosylation revealed consistent differences at ovulation. This study is the first approach to perform a comprehensive biochemical characterization of a mucus gel and secretion that included not only the proteins but also the mucins and their glycosylation.

MATERIALS AND METHODS

Reagents—All chemicals, unless otherwise stated, were purchased from Sigma.

Sample Collection and Preparation—A total number of 12 healthy, regularly cycling women, between 25 and 36 years of age, were enrolled in the study. Approval and informed consent were obtained from all women. The collection of the samples took place at the Department of Obstetrics and Gynecology, Sahlgren’s University Hospital, Gothenburg, Sweden. Cervical mucus samples (n = 36) were obtained by aspiration from the endocervix with a long tuberculin syringe. Samples from each woman were collected before, during, and after ovulation of one menstrual cycle. Ovulation was assessed by transvaginal ultrasonography and confirmed by measurement of midluteal serum progesterone levels. The cycle day is given from the 1st day of menstruation. The cervical mucus was collected in plastic tubes and directly stored at –80 °C. The amount of mucus from each patient was –100–150 μl before ovulation, 200–250 μl at ovulation, and 0–25 μl after ovulation. Each sample was dissolved with the same amount of a non-reducing sample buffer (4% SDS, 125 mM Tris-HCl buffer, pH 6.8, 30% glycerol, and 5% bromphenol blue). The solutions were reduced with DTT (final concentration of 20 mM) at 95 °C under magnetic stirring. Reduction was performed twice followed by incubation in 45 mM iodoacetamide for 1 h with magnetic stirring in room temperature.

One-dimensional SDS-PAGE and SDS-Agarose Composite Gel Electrophoresis for the Separation of Proteins and Mucins—Proteins and mucins of human cervical secretions were analyzed by one-dimensional SDS-PAGE, SDS-Agarose PAGE, and SDS-Agarose Composite Gel Electrophoresis for the Separation of Proteins and Mucins. Proteins and mucins from each patient were dissolved with the same amount of a non-reducing sample buffer (4% SDS, 125 mM Tris-HCl buffer, pH 6.8, 30% glycerol, and 5% bromphenol blue). The solutions were reduced with DTT (final concentration of 20 mM) at 95 °C under magnetic stirring. Reduction was performed twice followed by incubation in 45 mM iodoacetamide for 1 h with magnetic stirring in room temperature.

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One-dimensional SDS-PAGE and SDS-Agarose Composite Gel Electrophoresis for the Separation of Proteins and Mucins—Proteins and mucins of human cervical secretions were analyzed by one-dimensional SDS-PAGE and SDS-Agarose PAGE, respectively. For protein analysis, 1–11 μl of the samples were analyzed with discontinuous SDS-PAGE and SDS-Agarose PAGE, respectively. For protein analysis, 1–11 μl of the samples were analyzed with discontinuous SDS-PAGE and SDS-Agarose PAGE, respectively. For protein analysis, 1–11 μl of the samples were analyzed with discontinuous SDS-PAGE and SDS-Agarose PAGE, respectively. For protein analysis, 1–11 μl of the samples were analyzed with discontinuous SDS-PAGE and SDS-Agarose PAGE, respectively. For protein analysis, 1–11 μl of the samples were analyzed with discontinuous SDS-PAGE and SDS-Agarose PAGE, respectively. For protein analysis, 1–11 μl of the samples were analyzed with discontinuous SDS-PAGE and SDS-Agarose PAGE, respectively. For protein analysis, 1–11 μl of the samples were analyzed with discontinuous SDS-PAGE and SDS-Agarose PAGE, respectively.
Cervical Mucus Proteome and Glycome

capillary was connected to the column and to waste. The analytical column (17 cm × 50-µm inner diameter) consisted of a fused silica capillary packed with the same particles as the precolumn. During the injection, the split restrictor on the first T was closed while the other split was open, and a flow rate of 5 µl/min of 100% A (0.1% HCOOH) went through the trap column and out to waste. After 3 min the first split was opened and the second split was closed, and the gradient started (250 µl/min, 0.1% HCOOH, 0–50% ACN in 50 min) with a flow rate of ~100 nl/min through the column. The nano-ESI interface (Thermo Electron, Bremen, Germany) was modified in-house. The analytical column was sealed against a steel screen (1-µm pores, 50-µm thickness) and connected to the emitter (tapered fused silica, 20-µm inner diameter) in a ¼-inch through-bore union (Valco Instruments Co.). The voltage applied to the union was ±1.4 kV. The mass spectrometer was a hybrid linear ion trap-FT-ICR MS instrument equipped with a 7-tesla ICR magnet (LTQ-FT, Thermo Electron). The mass spectrometer was operated in the data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey MS spectra (from m/z 400 to 1600) were acquired in the FT-ICR instrument, and the three most intense doubly or triply charged ions in each FT scan were fragmented and analyzed in the linear ion trap.

Protein and Mucin Identification—Peak lists were extracted from raw data using the program "extract.msn.exe" (Thermo Finnegan). No smoothing was applied. Searches were performed using the search program Mascot (Version 2.1.0, Matrix Science, London, UK). The search parameters were set as follows: MS accuracy, 15 ppm; MS/MS accuracy, 0.5 Da; one missed cleavage allowed; fixed carbamidomethyl modification of cysteine; and variable modification of oxidized methionine. Searches performed on peptide extracts from protein bands from the SDS gel were performed as four merged searches of protein bands 1–15 and 13–28 (day 9) and 29–44 and 42–56 (day 14), respectively (see Fig. 2). The tandem mass spectra from the LC-FT MS/MS experiments were searched against the non-redundant protein sequence database downloaded from the National Center for Biotechnology Information (NCBI) May 10, 2005 containing 2,869,704 entries (www.ncbi.nlm.nih.gov/). Searches against the NCBI database revealed individual ion scores above 33 for 95% peptide identity significance level and above 40 for the 99% level. The minimum criteria for protein identification were set to two unique peptides above the 99% level. If the protein was identified twice (i.e. in both of the merged searches covering the day 9 or the day 14 sample) only the best peptide coverage was reported. Searches performed on mucin peptide extracts obtained from composite gels were performed as merged searches of mucin bands 1–5 and 6–10 corresponding to before and during ovulation, respectively. The MS data were searched against a mucin database compiled and made available by our group (www.medkem.gu.se/mucinbiology/databases). The human mucin database was Version 1.0 and contained 16 mucin sequences (complete sequences of MUC1, MUC2, MUC5B, MUC7, MUC4, MUC6, MUC12, MUC13, MUC15, MUC17, MUC19, and MUC20 and partial sequences of MUC3A, MUC3B, MUC5AC, and MUC16). The Mascot scoring system is influenced both by the number of sequence entries in the mucin database and the oligosaccharides were released with reductive β-elimination (13). Sample injection and capillary HPLC were performed by using the same instrumentation as for peptide analysis (see above). The samples were resuspended in 10 µl of water and injected onto graphitized carbon columns (150 × 0.32- or 0.25-mm inner diameter) packed in-house with 5-µm Hypercarb particles (Thermo-Hypersil, Runcorn, UK). Oligosaccharides were eluted with an H2O/ACN gradient containing 7 mEq NH4HCO3 (0–50% ACN 5–25 min followed by a 10-min wash step in 80% ACN). The flow rate was 5 µl/min and maintained by splitting the liquid flow from the pump with a fused silica restrictor (40 cm × 50-µm inner diameter). Mass spectra were recorded on a Finnegan LTQ linear ion trap mass spectrometer with an Ion Max ion source (Thermo Finnegan) in negative ion mode. The capillary temperature was 340 °C, the capillary voltage was 31 V, and the electrospray voltage was 3.2 kV. For MS/MS experiments, the normalized collision energy was 20% with an activation time of 30 ms. Mass spectrometry was performed with four scan events: full scan with mass range m/z 300–2000 followed by successive MS/MS scans after collision-induced fragmentation for the three most intense ions in each full scan.

RESULTS

Proteins in the Cervical Mucus—The mucus of the cervical opening was collected from 12 healthy women from before, during, and after ovulation by gentle aspiration to minimize the number of epithelial and blood cells. The volumes of aspirated mucus varied during the menstrual cycle as shown in Fig. 1 (total volume). The DTT-reduced mucus was alkylated and separated on 3–15% SDS-PAGE gels, and the protein bands were revealed by silver stain (Fig. 1). A few intense and a number of less intense bands were observed including a strongly stained band in the top of the gel. This latter band consists of the mucins, which were analyzed separately on composite gels (see below). Mucins were stained more weakly than less glycosylated proteins by silver stain, indicat-
ing that the proteins found in the mucin band were dominating the cervical mucus. The general protein profile before, during, and after ovulation from a single individual was relatively constant, but some exceptions were noticed as exemplified by cycle days 7 and 12 compared with day 19 of subject 1. However, comparing the analyses of all 12 women revealed no consistent alterations of the protein pattern over the menstrual cycle. To further analyze the nature of the proteins, mucus samples from before and during ovulation were studied by proteomics and are presented from one individual (Fig. 2). The samples were separated by SDS-PAGE and developed with Coomassie Blue, and the whole lane (except the mucin band at the top) was sliced in 28 pieces including the main distinct bands as indicated in Fig. 2. The trypsin-digested bands were analyzed by nano-LC-FT-MS/MS for protein identification. The results are given in Supplemental Table S1. The strongly stained bands 14 and 43 were identified as the poly-Ig receptor, and bands 16 and 45 were identified as albumin.

**Cervical Mucins**—The DTT-reduced and alkylated cervical mucins were analyzed by gradient AgPAGE that resolves the large mucins. Alcian blue, a stain that binds negatively charged oligosaccharides, revealed the mucin bands (Fig. 3). The sizes of the mucins were 2–3 MDa. The profiles of the mucins from the three menstrual time points and from the different individuals resembled each other. The gels revealed several ladder-like band patterns with a decreasing intensity as the electrophoretic mobility increased. Ten mucin bands from before and during ovulation of several individuals were isolated as illustrated in Fig. 4 and analyzed after trypsin digestion by nano-LC-ICR MS and MS/MS. The results were subjected to merged searches against a curated in-house human mucin database (www.medkem.gu.se/mucinbiology/).
databases). We identified three gel-forming and two transmembrane mucins (Table I and Supplemental Table S3). The MUC5B was the most abundant mucin as judged from the total number of identified peptides (83 peptides). This corresponds to a peptide coverage of 16% of the whole protein. This is a high percentage as most peptides from mucins are glycosylated and thus cannot be identified from peptide databases. The MUC5B peptides originated from its Cys-rich N and C termini as well as the repeated Cys-D domains interspersed between the mucin domains. Peptides for the MUC5B mucin were found in all mucin bands (Fig. 4, bands 1–10) revealed by AgPAGE.

The MUC5AC mucin has a size and structure similar to those of MUC5B and was identified with 33 peptides (32 and 26 peptides, before and during ovulation, respectively). The lower peptide coverage of MUC5AC compared with that of MUC5B suggests that MUC5AC is less abundant. The MUC5AC mucin was identified in all analyzed mucin bands (Fig. 4), and the peptides were found spread out all over the protein except for the mucin domains. A third gel-forming mucin, MUC6, also was identified. This mucin was only identified in the samples collected before ovulation. Only 10 peptides were found, indicating a lower abundance than for the others. The MUC6 peptides were found in the three upper, slowest migrating bands (Fig. 4, bands 1–3). Most peptides originated from the N-terminal half of the protein except for two peptides from the C terminus.

In addition to the gel-forming mucins, two transmembrane mucins were identified. A total of 31 identified peptides originated from MUC16 with 27 and 20 peptides identified from before and during ovulation, respectively. These peptides were found in the three lower, faster migrating mucin bands on the composite gel (Fig. 4, bands 3–5 and 8–10). The peptides were mainly from the less glycosylated C-terminal half of the protein. Finally two peptides of the MUC1 mucin were identified at both time points; these were also from its C-terminal end.

Glycosylation of the Cervical Mucins—The O-glycosylation of the cervical mucus before, during, and after ovulation was analyzed from six individuals. Mucins separated by AgPAGE were blotted to PVDF membranes, and the O-glycans were released and analyzed with negative mode LC-MS and -MS/MS. Identified oligosaccharide sequences are listed in Supplemental Table S4.

FIG. 5. Total ion spectra of the O-linked oligosaccharides from the Alcian blue-stained bands of mucins collected before ovulation (day 9, top) and during ovulation (day 14, bottom). The oligosaccharides were analyzed with capillary LC-MS and -MS/MS. Identified oligosaccharide sequences are listed in Supplemental Table S4.

<table>
<thead>
<tr>
<th>Sample</th>
<th>MUC5B</th>
<th>MUC5AC</th>
<th>MUC6</th>
<th>MUC16</th>
<th>MUC1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 6</td>
<td>5</td>
<td>75</td>
<td>3</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Day 14 (ovulation)</td>
<td>7</td>
<td>73</td>
<td>6</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Protein size (kDa)</td>
<td>590</td>
<td>&gt;550&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&gt;2200&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2352</td>
<td>122&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Six identified peptides in common.
<sup>b</sup> Peptide significance identity level.
<sup>c</sup> Approximate size; sequencing has not been completed.
<sup>d</sup> Approximate size; polymorphic variants occur.
similar, dominated by sialylated oligosaccharides. The major peak at \( m/z \) 675 of these spectra (see Fig. 5) consisted of two sialylated isoforms of a trisaccharide, Gal-3(\( \text{NeuAc-6} \))GalNAcol (the 6-linked branch attached to the GalNAcol are in bold) and a less abundant NeuAc-Gal-GalNAc sequence (Fig. 6). In contrast, the oligosaccharide profiles of the cervical mucins collected during ovulation were dominated by neutral oligosaccharides (Fig. 5). Four oligosaccharide isoforms, one at \( m/z \) 749 with the sequence Gal-3(\( \text{Gal-4GlcNAc-6} \))GalNAcol and three neutral fucosylated pentasaccharides at \( m/z \) 895 with the sequences Fuc-Gal-3(\( \text{Gal-GlcNAc-6} \))GalNAcol, Gal-3(\( \text{Fuc-Gal-4GlcNAc-6} \))GalNAcol, and Gal-3(\( \text{Fuc-(Gal-GlcNAc-6}) \)GalNAcol, were increased relative to other oligosaccharides during ovulation (Fig. 6). A similar trend was present in the oligosaccharide profiles obtained from all six individuals. The separation of the mucins into several bands with different electrophoretic mobility as revealed in Fig. 3 could not be explained by different glycosylation as the glycans from the same individual appeared to be identical (results not shown).

**DISCUSSION**

In the present study, we determined the proteome and glycome of the cervical mucus that protects the entry into the uterine cavity. Mucus is dominated by its major constituent, the mucins, which give the mucus most of its gel-like properties. This is also the case for the cervical mucus where the gel-forming mucin MUC5B is the major component together with MUC5AC. As the major constituent of the mucins is their O-glycans, our analysis of the mucin glycome also reflects a major part of the cervical mucus glycome. For a complete picture of the cervical mucus proteome, proteins other than the mucins were studied.

A total number of 194 proteins were identified, many present in the cervical secretions collected both before and during ovulation. The identified proteins covered a broad range of protein functional classes associated with diverse biological processes and of both cellular and secreted nature. A major goal of the present study was to identify alterations in the cervical mucus composition during the menstrual cycle. When other proteins than mucins were separated by gel electrophores, the silver-stained protein profiles from different cycle days revealed a common pattern with some differences between the different individuals or cycle days. The major reason for these differences is the problem of collecting pure mucus. The mucus normally contains epithelial cells, but we also observed that the collection procedure in itself affects the amount of epithelial cells and blood. During the initial phase of the project, the common brush method was used to collect the material. However, this gave a large amount of cervical epithelial cells and blood. To improve the quality of the analyzed material, the samples were collected by gentle suction. Also this gave a variable number of epithelial cells and sometimes small amounts of erythrocytes, although much less than with the brush method. The blood is reflected in the presence of \( \beta \)-globin, and epithelial cells are reflected in the high number of intracellular and membrane components identified in the proteome analyses. The variable amount of epithelial cells caused most of the differences observed upon gel electrophoresis and proteomics, although some differences might be due to individual variability.

The cervical mucus normally also contains extracellular fluid as reflected in the presence of albumin, one of the more abundant components in all analyzed subjects. Many of the identified proteins are normal components of serum and extracellular fluid; others are known components of mucus. Examples of mucus constituents are lactotransferrin (lactoferrin), poly-Ig receptor, and IgA, all found with high scores. Other proteins are also likely to be directly secreted into the
mucus as for example DMBT1 and the Fc fragment of IgG-binding protein (FCGBP). Some of the proteins have not been described previously in cervical mucus but have been described in salivary secretions (16). These include amylase, cystatins, and calcium-binding protein S100. The LPLUNC1 protein (von Ebner salivary gland protein) has only been identified previously in saliva. Thus, it seems clear that cervical mucus contains a number of cellular, serum, and specific mucus proteins. After studying a number of gel electrophoresis protein patterns from different individuals and at different time points during the menstrual cycle as well as the comparative proteome analyses, we were not able to identify any obvious alterations among the ordinary proteins.

Turning to the mucins, we identified five different mucins: three gel-forming (MUC5B, MUC5AC, and MUC6) and two membrane-bound mucins (MUC1 and MUC16). These mucins, except MUC16, have been described previously in the female reproductive epithelia by transcriptional analysis of mucin gene expression (5). The MUC5B mucin has been found in the cervix throughout the menstrual cycle as determined by antibodies directed against the protein backbone (4), and both MUC5B and MUC5AC are present in the cervical mucus plug of pregnant women (17). Our results support the previous notion that MUC5B is the major mucin of the cervical mucous. However, we also found a relatively high number of peptides for the MUC5AC mucin, indicating that this mucin also is present in substantial amounts (Table I). None of the mucin bands in the AgPAGE could be attributed to a single mucin. This observation is in line with the previous notion of increased MUC5B expression at ovulation (4). The functional differences of these three gel-forming mucins remain unclear. MUC5B and MUC6 are both typical for glands, whereas MUC5AC is normally found in goblet cells. The functional importance of these individual mucins in the cervical mucus is currently not understood.

Two transmembrane mucins, MUC1 and MUC16, were identified. The MUC1 mucin has been studied as a component of the uterine epithelium (18) and in relation to cancer, for example breast cancer and pancreas cancer (6). The MUC1 expression in the uterus is under steroid hormone control and peaks at the progesterone-dominated secretory phase. The functional role of uterine MUC1 as well as MUC1 in other tissues is not well understood. However, some insight has been obtained from studies of its cytoplasmic tail that is involved in intracellular signaling (19). Recently the structure of the extracellular SEA domain has provided further insight into its potential function (20). The cleaved SEA domain acts as breakpoint, probably for mechanical forces, protecting the cell, at the same time as it may be a mechanosensor. Both the peptides identified for MUC1 are located in the N-terminal part outside of the cleavage in the SEA domain. This could suggest that the outer part has been separated from the membrane anchor. It is less likely that the cervix mucus MUC1 is from the alternatively spliced MUC1/SEC as this protein lacks one (QGGFLGLSNIK) of the two identified peptides (Supplemental Table S3). Also the MUC16 mucin, identified in our cervical samples, has one of at least 62 SEA domains that might be cleaved (20). The MUC16 mucin was recently cloned and shown to be an enormous mucin with more than 22,000 amino acids (21, 22). Most of the peptides identified (Supplemental Table S3), except four, occur several times in the MUC16 sequence. This mucin is the ovarian cancer antigen CA125 (23) as detected by the specific antibody OC125 (21). This antibody has been used on cervical mucus, and its positive staining suggested the presence of the MUC16 mucin (24). Also in our experiments, the OC125 antibody stained a Western blot band confirming the presence of the MUC16 mucin (results not shown). Little is known about the normal function of the MUC16 mucin and its role in the cervical mucus.

We did not identify peptides corresponding to the membrane-spanning mucin MUC4 shown previously to be expressed at high mRNA levels in cervix, levels equivalent to MUC5B (3). The reason for this discrepancy remains unclear. One possible explanation could be that the MUC4 transcript is not translated into protein. Mucins are also difficult to identify with proteomics as most of their peptides are glycosylated and thus not identified in the protein databases. Still our results suggest that MUC4 is not a major mucin in the cervix. The presence of multiple bands with different electrophoretic mobility on the composite gel of the reduced mucins from the same cycle day remains unexplained (Fig. 3). The proteomics results suggest that all analyzed mucin bands contain mixtures of mucins. MUC5B and MUC5AC peptides were found in all analyzed bands. Similar patterns with multiple bands on composite gels have also been observed in for example human bronchial epithelial cell mucins (25) and salivary mucins (26), the latter explained by the presence of glycoforms. However, our oligosaccharide analysis of the different cervix mucin bands suggests that these have similar glycosylation. One possible explanation for the presence of multiple mucin bands may be that multimeric molecules could be generated by the formation of non-reducible bonds between protein monomers as described previously (27). Cleavages in the gel-forming mucins as well as a combination of different mucins and glycosylation could add up to the multiple mucin bands observed on the composite gel.

The O-glycans of the cervical mucins revealed more than 50 different neutral, sialylated, and sulfated oligosaccharides. Our results disclosed a much more diverse cervical mucin glycosylation than observed previously. Using traditional biochemical approaches, others have previously isolated and sequenced eight neutral and sialylated oligosaccharides from cervical secretions collected during ovulation (28–30). We also identified these eight components, although we were not always able to assign the exact position of the sialic acid residue. The subjects analyzed here were of blood group O type, and most of them were positive for secretor and Lewis as reflected in the identified oligosaccharide structures. Typ-
For these mucin oligosaccharides are the high degree of fucosylation (Supplemental Table S4) and the presence of blood group H epitopes. It is thus obvious that the blood group status of the woman is reflected in the cervical mucus, something that could affect susceptibility to infections. In fact, it has been observed that secretor-negative women that lack a functional FUT2 fucosyltransferase are more prone to develop recurrent *Candida albicans* vaginitis (31).

The major alterations observed during the menstrual cycle were in the mucin O-glycosylation. The O-glycans were virtually identical in the mucins before and after ovulation but were substantially different at ovulation. The relative abundance of neutral oligosaccharides versus acidic ones thus increased during ovulation. Some of the major glycans that were altered are illustrated in Fig. 6. The characteristic alterations observed were a decrease in both NeuAc2–6 attached to the GaINAc and the NeuAc2–3 to Gal–GaINAc at ovulation with a concomitant increase in the Core 2 glycan (Gal–1–3GlcNAc1–6GalNAc) typically substituted with fucose. The GlcNAc6GalNAc was often also sulfated at the time of ovulation. Thus major alterations were probably an increase in the Core 2 GlcNAc transferase as well as a decrease of sialyltransferases. Previous studies using histochemical and simpler biochemical methods have also suggested cyclic glycosylation variation over the ovulation period (8, 10, 32, 33).

The present observations bring the understanding of the composition and the cyclical changes of the cervical mucus forward. The mucus was similar before and after ovulation but differed in the midcycle. We did not observe any differences in the large number of small proteins found in the mucus. Instead the most remarkable difference was a shift in the mucin glycosylation. The physiological importance of this shift is currently not understood but could well mean altered mucus properties by the physicochemical changes or altered interactions with lectins in the mucus. The mucin glycosylation at ovulation could also promote sperm penetration by its low sialic acid content. Although the composition of the mucin seems to be relatively constant except for the mucin glycosylation, other alterations in for example the mucins could contribute to the altered mucus properties. One such possible example is the recently observed autocatalytic cleavage of the MUC5AC mucin (34), something that also needs to be studied in cervical mucus.

Acknowledgments—We gratefully acknowledge the assistance of Hasse Karlsson and Thomas Larsson (Proteomics Core Facility). Tiange Lang is acknowledged for work with compiling the mucin database.

This work was supported by The Swedish Research Council (Grant 7461 and for the mass spectrometer), The Postgenomic Research and Technology Program in Western Sweden (Swegene), The Wallenberg Foundation, Ingabritt and Arne Lundberg Foundation, and Swedish Foundation for Strategic Research-The Mucosal Immunobiology and Vaccine Centre (MIVAC). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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REFERENCES


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