Mucin gene expression in human male urogenital tract epithelia

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BACKGROUND: Mucins are large, hydrophilic glycoproteins that protect wet-surfaced epithelia from pathogen invasion as well as provide lubrication. At least 17 mucin genes have been cloned to date. This study sought to determine the mucin gene expression profile of the human male urogenital tract epithelia, to determine if mucins are present in seminal fluid and to assess the effect of androgens on mucin expression. METHODS AND RESULTS: Testis, epididymis, vas deferens, seminal vesicle, prostate, bladder, urethra and foreskin were assessed for mucin expression by RT–PCR (for 14 mucin genes) and immunohistochemistry (nine antibodies for five mucins). Epithelia of the vas deferens, prostate and urethra expressed the greatest number of mucins, each with mRNA for between 5 and 8 mucins. Except for MUC20 in epididymis, mRNA for MUC1 and MUC20, both membrane-associated mucins, was detected in all tissues analysed. By comparison, MUC6 was more restricted in expression, being primarily detected in seminal vesicle. MUC1, MUC5B and MUC6 were detected in seminal fluid samples by immunoblot analysis. Androgens had no effect on mucin expression in cultured human prostatic epithelial cells. CONCLUSIONS: Each region of urogenital tract epithelium expressed a unique mucin gene repertoire. Secretory mucins are present in seminal fluid, and androgens do not appear to regulate mucin gene expression in prostatic epithelial cells in culture.

Key words: male reproductive tract epithelia/male urogenital tract/MUC/mucin

Introduction

The wet-surfaced epithelia of the body, including those of the urogenital, gastrointestinal and respiratory tracts, and the ocular surface, are the primary sites of pathogenic infection. Wet-surfaced epithelia, in addition to providing defence mechanisms against infection, must be lubricated to prevent luminal surfaces from adhering to one another. Molecules that provide both pathogen barrier and lubricating, disadhesive functions to the wet-surfaced epithelia of the body are large, high molecular weight, hydrophilic glycoproteins known as mucins (for review, see Jeffery and Li, 1997; Desouza et al., 1998; Lagow et al., 1999; Corfield et al., 2001; Gipson and Argueso, 2003; Gipson et al., 2004). Mucins by definition have tandem repeats of amino acids in their protein backbone rich in serine and threonine, which provide sites for O-linked glycosylation. Carbohydrates usually make up a majority of the mass of the mucin molecule (Gendler and Spicer, 1995; Moniaux et al., 2000). To date, at least 17 mucin genes have been cloned (Gendler et al., 1987; Gum et al., 1989; Gum et al., 1990; Lan et al., 1990; Pochet et al., 1991; Bobek et al., 1993; Dufosse et al., 1993; Toribara et al., 1993; Arias et al., 1994; Meezaman et al., 1994; Shankar et al., 1994; Guyonnet Duperat et al., 1995; Williams et al., 1999; Pratt et al., 2000; Williams et al., 2001; Yin and Lloyd, 2001; Gum et al., 2002; Pallesen et al., 2002; Chen et al., 2004; Higuchi et al., 2004): MUC1–4, 5AC, 5B, 6–13, 15–17, 19 and 20. On the basis of sequencing data, two classes of mucins have been identified. These include the membrane-associated mucins (MUC1, 3, 4, 11/12, 13, 15, 16, 17 and 20) and the secreted mucins. The latter category includes both the large gel-forming (MUC2, 5AC, 5B, 6 and 19) and small soluble mucins (MUC7 and 9).

Membrane-associated mucins have hydrophilic domains towards the carboxy terminus of the protein that allow them to span the cell membrane. The extracellular domain of membrane-associated mucin molecules may extend up to 500 nm from the epithelial cell surface (Bramwell et al., 1986), forming a dense glyocalyx along the apical surface of the epithelia, which is the cells’ protective barrier. Experimental data indicate that the extracellular domain of membrane-associated mucins also provides a disadhesive character to the apical, luminal surfaces of epithelia (Hilkens et al., 1992; Litgenberg et al., 1992; Komatsu et al., 1997). The studies further indicate that the negatively charged carbohydrate residues on the mucin protein backbone provide the disadhesive character (Hilkens et al., 1992; Litgenberg et al., 1992). The extracellular domain of membrane-associated mucins can be detected in mucosal
secretions (Ellingham et al., 1997; Jumblatt et al., 1999; Pflugfelder et al., 2000; Zhao et al., 2001; Argueso et al., 2002; Spurr-Michaud et al., 2004) as well as in sera of breast tumour (Burchell et al., 1984; Linsley et al., 1986) and ovarian tumour (Yin et al., 2002) patients, indicating that the extracellular domain is shed from apical surfaces (Rossi et al., 1996) or that splice variants are secreted, in which the membrane-spanning and cytoplasmic domains are not translated (Gendler, 2001; Moniaux et al., 2001). The shed membrane-associated mucins may contribute to protection/lubrication of the epithelia in both soluble and membrane-tethered forms. Recent data indicate that membrane-associated mucins are multi-functional molecules, providing not only barrier and lubrication functions but also signal transduction through their juxta-membrane regions (Pandey et al., 1995; Yamamoto et al., 1997; Carraway et al., 2002; Ren et al., 2002). Several membrane-associated mucins have epidermal growth factor (EGF)-like domains near their membrane-spanning domain, and phosphorylation sites have been detected in the MUC1 cytoplasmic tail (Zrihan-Licht et al., 1994; Pandey et al., 1995; Quin and McGuckin, 2000).

The secreted mucins, gel-forming and small soluble, are produced by epithelial goblet cells and associated submucosal glands. The common features of gel-forming mucins are cysteine-rich D domains on either side of long domains of tandem repeats. Three D domains are located in the amino terminal region of the protein and one in the carboxy region (Perez-Vilar and Hill, 1999). MUC6, however, lacks the carboxy D domain. The D domains provide sites for homomultimerization of gel-forming mucin molecules; the large homo-multimers provide the viscosity of the mucosal secreta. The small soluble mucins lack D domains and subsequently remain as monomers (Bobek et al., 1993).

Although mucins of the female reproductive tract have been studied (Carson et al., 1998; Gipson, 2001), little is known about the mucin gene repertoire of the male urogenital tract. As assayed by in situ hybridization (ISH) and immunohistochemistry, ejaculatory epithelial ducts and seminal vesicles expressed MUC6, whereas the prostate and bladder samples tested did not (Bartman et al., 1998; Leroy et al., 2003). In other male urogenital tissues, MUC1 mRNA and protein have been detected in human testis and epididymis (Franke et al., 2001), and MUC1, 3 and 4 have been reported in the normal urothelium (N’Dow et al., 2000). Mucin gene expression in the human prostate, however, is controversial. In contrast to the MUC6 studies cited above (Bartman et al., 1998; Leroy et al., 2003), Northern blot and immunostaining assays detected MUC1 and 6 in the human prostate (Ho et al., 1993; Gold et al., 1994). Furthermore, Ho et al. (1993) did not detect MUC2 or 3 in the prostate, whereas Durrant et al. (1994) reported MUC2 expression using an antibody to the tandem repeat of the mucin. A comprehensive analysis of mucin gene expression in the human testis, epididymis, vas deferens, seminal vesicle, prostate, bladder, urethra and foreskin has not been reported. Inconsistencies such as those reported for MUC2 and MUC6 expressions in the prostate (Ho et al., 1993; Durrant et al., 1994) validate assaying human specimens for both mucin mRNA and protein. In addition, there have been no reports on specific mucin components of seminal plasma. Similarly, androgen regulation of mucin gene expression in male urogenital tract epithelia has not been studied. A comprehensive analysis of mucins in the male urogenital tract will provide a baseline for future studies that aim to clarify which mucins may be involved in the various activities of the different regions of the male urogenital tract, where they may contribute to protection against infections from sexually transmitted pathogens, provide disadhesive functions to facilitate sperm passage and provide lubrication to maintain luminal patency.

Materials and methods

Tissue and seminal plasma acquisition and preparation

All discarded human tissue specimens of surgical and post-mortem origin collected from the human male urogenital tract were obtained in accordance with good clinical practice. Institutional Review Board and informed consent regulations of the Schepens Eye Research Institute and the Brigham and Women’s Hospital, and the doctrines of the Declaration of Helsinki. Normal discarded tissues were collected from the urogenital tract during autopsy of male patients or as surgical specimens from prostatectomy, orchietomy, malignancy of the bladder, vasectomy and circumcision. Tissues collected included testis, epididymis, vas deferens, seminal vesicle, prostate, bladder, urethra and foreskin. The tissues were snap frozen in liquid nitrogen for RNA isolation, frozen in optimum cutting temperature (OCT) compound (IMEB, Inc., San Marcos, CA, USA) or fixed in 4% paraformaldehyde for immunofluorescence (IF) microscopy within 45 min of surgical removal or 38 h post-mortem. On average, three to seven specimens were assayed for mucin mRNA and three to nine specimens for mucin protein, whereas only one to two specimens of vas deferens and epididymis were available (discarded epithelial specimens from these tissues were difficult to collect).

Five semen samples were collected for immunoblot analysis from five male partners of women who had been pregnant within the previous 2 years. After 48 h of sexual abstinence, semen samples were collected by masturbation and submitted for semen analysis. Samples were diluted at 1:1 with sterile phosphate-buffered saline (PBS), mixed thoroughly and centrifuged for 10 min at 400 × g. Seminal plasma was removed, aliquoted and stored at −70°C.

Cell culture

We sought to characterize non-cancerous prostatic epithelial cells for mucin gene expression to identify potential transcriptional regulators of mucin expression. The primary human, adult prostatic epithelial cells used were PrEC (Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA). Cells were grown in Clonetics™ Prostate Epithelial Growth Media (PrEGM; Cambrex Bio Science Walkersville, Inc.) containing bovine pituitary extract, insulin, hydrocortisone, gentamicin sulphate amphotericin-B, retinoic acid, transferrin, triiodothyronine, epinephrine and human recombinant EGF, as per the manufacturer’s instructions. Cells were cultured in six-well plates at 2.5 × 10⁴ cells/cm² at 37°C in a 5% carbon dioxide atmosphere to confluence. To assess the potential regulatory effects of androgens and serum on mucin gene expression, we supplemented the media with one of the following: 10% (v/v) calf serum (Invitrogen, Rockville, MD, USA), 10⁻⁶ M dexamethasone, 10⁻⁸ M dihydrotestosterone (DHT) (Steraloids, Inc.; Newport, RI, USA) or 1 nM or 5 nM mibolerone, a non-metabolizable androgen (BioMol Research Laboratories, Inc.; Plymouth Meeting, PA, USA). Dexamethasone, DHT and mibolerone were dissolved in ethanol (≤0.01% v/v final concentration). Duplicate wells for each treatment condition, including an untreated control group, were harvested at 1, 3 and 6 days. The media were changed every 48 h.
RNA isolation and reverse transcription
Total RNA was extracted from pulverized, frozen tissues, using TRIZol reagent (Invitrogen) in accordance with the manufacturer’s recommended protocol. Cell culture lysates were homogenized in 1× Lysis Buffer (PE-Applied Biosystems, Foster City, CA, USA), and total RNA was isolated using the ABI Prism 6100 Nucleic Acid PrepStation (Applied Biosystems User Guide, 2001). As previously described (Argueso et al., 2002), 2 µg aliquots of total RNA were treated with DNase I (Amplification Grade; Invitrogen) and reverse transcribed using random hexamer primers and Superscript II reverse transcriptase (Invitrogen), as per the manufacturer’s instructions.

PCR
RNA isolated from tissue specimens was assayed by conventional RT–PCR at 35 cycles, to look for expressions of MUC1, 2, 3, 4, 5AC, 5B, 6, 7, 13, 15, 16, 17, 19 and 20, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a quality control. PCR primers and amplification parameters for the mucin genes are indicated in Table I (Finkbeiner et al., 1993; Bernacki et al., 1999; Argueso et al., 2003). MUC3 primers used in this screening recognized a homologous region in both MUC3A and MUC3B genes. Studies of the incompletely characterized mucins, MUC8, 9 and 11, were not conducted. Real-time PCR-compatible primers (TaqMan chemistry, Applied Biosystems) for membrane-associated mucin MUC15 (Palleisen et al., 2002) were designed (Table I) using Primer Express software (Applied Biosystems), and nucleotide database searches using BLASTN (http://www.ncbi.nlm.nih.gov/blast/, provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD, USA) verified the specificity of the MUC15 primer set. The identity of the purified 81-bp MUC15 PCR product was confirmed by the DNA Sequencing Center for Vision Research, the Ocular Molecular Genetics Institute of the Massachusetts Eye and Ear Infirmary (Boston, MA, USA).

Androgen receptor expression in RNA isolated from PrEC cells was determined by RT–PCR, using primers and amplification parameters as previously described (Table I) (Lau et al., 2000). Samples lacking complementary DNA (cDNA) were run in each assay as negative controls, and cDNA from different tissues known to express one of the mucin genes or from human prostatic tissue, known to express the androgen receptor, were run as positive controls. Amplified products were run on a 2% agarose gel and visualized with ethidium bromide.

Real-time PCR
Relative mucin gene expression in the PrEC cell line was assayed with double-labelled fluorogenic primers and probes (TaqMan; Applied Biosystems), as previously described (Argueso et al., 2002), using a sequence detection system (ABI Prism 7900HT; Applied Biosystems). MUC1, MUC4, MUC5AC (Table I) and GAPDH PCR primers and probes used in this study have been reported (Argueso et al., 2002). Equivalent PCR amplification efficiencies for GAPDH, the endogenous control and the target mucin genes were confirmed.

The ΔCt method (Applied Biosystems) was employed for the relative quantification of mucin gene expression (Argueso et al., 2002; Gipson et al., 2003). Quantitative mRNA expression of each mucin gene amplified in the cell line was expressed relative to the amount of MUC1 mRNA present in a calibrator sample (ΔΔCt method), namely the untreated control culture group of each time point assayed. Samples were amplified in triplicate in a 50 µl total volume reaction for 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. Controls (no template) included in each real-time PCR assay confirmed that the amplification reagents were not contaminated with DNA. Real-time PCR results were statistically compared using the Fisher Protected Least-Significant Difference (Fisher’s PLSD) test (StatView, Version 5.0; SAS Institute, Cary, NC, USA). P < 0.05 was considered significant.

IF microscopy
Morphological characteristics of each of the male urogenital tract tissues were analysed on haematoxylin- and eosin-stained sections. Mucins were immunolocalized in the testis, epididymis, seminal vesicle, prostate, bladder, urethra and foreskin by IF microscopy as previously described (Gipson et al., 1992; Inatomi et al., 1995). Vas deferens tissue was not available for IF analysis. Tissues were incubated for 1 h at room temperature in primary antibody. Antibodies and their dilutions used are indicated in Table II. All antibodies, with the exception of the MUC4 antibody designated 528, have been previously described. The antibody 528 was made to a synthetic peptide (SSIVPGTFHPTLSEAC) from the deduced amino acid sequence of a MUC4 genomic clone (Gipson et al., 1999). Peptides were synthesized by solid-phase procedure (F moc chemistry) and then glutaraldehyde-conjugated to keyhole limpet haemocyanin in the Peptide Synthesis Core of the Reproductive Endocrine Sciences Center, Massachusetts General Hospital. Antibodies against the conjugated 528 peptide were produced in chickens by Avian Antibodies, Inc. (Carlisle, MA, USA) and affinity purified before use.

Sections lacking primary antibody were run as negative controls. Sections were incubated in secondary antibody in the fluorescein-conjugated donkey-anti-mouse immunoglobulin (IgG) or donkey-anti-chicken IgY (both at 1:50 dilution from Jackson Immuno Research; West Grove, PA, USA) and were cover-slipped with Vectashield, a propidium iodide-containing antifade mounting medium that also acts as a nuclear counterstain (Vector Laboratories, Burlingame, CA, USA) (Argueso et al., 2002).

Sections incubated with the MUC6 primary antibody, CLH5, were pretreated with neuraminidase as previously reported (Gipson et al., 1992). Antigen retrieval (15 min at 97°C in 10 mM citrate buffer, pH 6.0) was necessary before incubation with the commercially available MUC4 antibody, 1G8.

Agarose gel electrophoresis and immunoblot analysis
Total protein (125 µg) from seminal plasma was denatured and separated under reducing conditions in a 1% (w/v) agarose gel in electrophoresis buffer [Tris/Glycine/sodium dodecyl sulphate (SDS)], a modification of Thornton et al. (2000). Appropriate controls were also included in the assays to ensure the specificity of the protein detected. The proteins were then vacuum blotted transferred to a nitrocellulose membrane, blocked with 5% non-fat dry milk (w/v) in Tris-buffered saline with 0.1% Tween 20 or PBS, and probed with the following antibodies: 214D4 (MUC1), 8G7 (MUC4), CLH2 (MUC5AC), 799W (MUC5B) and CLH5 (MUC6) (Table II). Following incubation with peroxidase-conjugated goat-anti-mouse IgG1 (MUC1, 4, 5AC and 6) or anti-chicken IgY (MUC5B) secondary antibody, binding was detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

Results
To determine the mucin profile for native human male urogenital tract epithelia, we analysed human tissue from multiple individuals, as available, for mRNA and protein.
Table 1. Primers used for PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sets</th>
<th>Product Size</th>
<th>Traditional amplification</th>
<th>PCR parameters</th>
<th>Reference</th>
<th>GenBank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUC1</td>
<td>F: 5'-GTG CCC CCT AGC AGT ACC G-3' R: 5'-GAC GTG CCC CTA CAA GTT GG-3'</td>
<td>104 bp</td>
<td>1 cycle: 94°C for 1 min; 30 cycles: 94°C for 10 min;</td>
<td>95°C for 10 min; 95°C for 15 s; 60°C for 1 min</td>
<td>Argueso et al. (2002)</td>
<td>NM_002456</td>
</tr>
<tr>
<td>MUC2</td>
<td>F: 5'-CAAG CAC CGC CCA TTG AGT G-3' R: 5'-CAC CGC CCA TTG AGT G-3'</td>
<td>441 bp</td>
<td>1 cycle: 94°C for 5 min; 35 cycles: 94°C for 1 min;</td>
<td>94°C for 5 min; 94°C for 1 min; 60°C for 1 min; 72°C for 1 min</td>
<td>Finkbeiner et al. (1993)</td>
<td>NM_002457</td>
</tr>
<tr>
<td>MUC3</td>
<td>F: 5'-CTT CAT TGC AAA CTT CAC TCG-3' R: 5'-AGC CCA CAT TTT CTG TAC TG-3'</td>
<td>234 bp</td>
<td>1 cycle: 94°C for 2 min; 35 cycles: 94°C for 1 min;</td>
<td>94°C for 2 min; 94°C for 1 min; 55°C for 2 min; 72°C for 3 min.</td>
<td>Bernacki et al. (1999)</td>
<td>AF007194</td>
</tr>
<tr>
<td>MUC4</td>
<td>F: 5'-GCA GAC TCA GTG TTA ACT C-3' R: 5'-ATG GTG CCG TTG TAA TTT GTT GT-3'</td>
<td>101 bp</td>
<td>1 cycle: 94°C for 2 min; 35 cycles: 94°C for 1 min;</td>
<td>94°C for 2 min; 94°C for 1 min; 55°C for 2 min; 60°C for 1 min</td>
<td>Argueso et al. (2002)</td>
<td>AF058803</td>
</tr>
<tr>
<td>MUC5C</td>
<td>F: 5'-TCC ACC ATA TAC CGC CAC AGA-3' R: 5'-TGG ACC GAG AGT CAC TGT CAA G-3'</td>
<td>103 bp</td>
<td>1 cycle: 94°C for 2 min; 35 cycles: 94°C for 1 min;</td>
<td>94°C for 2 min; 94°C for 1 min; 55°C for 2 min; 60°C for 1 min</td>
<td>Argueso et al. (2002)</td>
<td>Z48314</td>
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<tr>
<td>MUC5B</td>
<td>F: 5'-GAC ATT GAC CCG TTC TCC CAG-3' R: 5'-GAG ATT CCC AAG CCG TGC ATG-3'</td>
<td>405 bp</td>
<td>1 cycle: 94°C for 5 min; 35 cycles: 94°C for 40 s;</td>
<td>94°C for 5 min; 94°C for 40 s; 58°C for 1 min; 72°C for 1 min</td>
<td>Gipson et al. (1999)</td>
<td>U78553</td>
</tr>
<tr>
<td>MUC6</td>
<td>F: 5'-AGG AGG AGA TCA CGT TCA AG-3' R: 5'-TGG CAT CTG CAC ATC TTG AG-3'</td>
<td>303 bp</td>
<td>1 cycle: 94°C for 2 min; 35 cycles: 94°C for 40 s;</td>
<td>94°C for 2 min; 94°C for 40 s; 64°C for 1 min; 72°C for 2 min</td>
<td>Gipson et al. (1999)</td>
<td>U97698</td>
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<tr>
<td>MUC7</td>
<td>F: 5'-AAA TAG CAG TGT GGT CAA CC-3' R: 5'-GCA CTG TGC ATG CAC ATT AG-3'</td>
<td>901 bp</td>
<td>1 cycle: 94°C for 2 min; 35 cycles: 94°C for 1 min;</td>
<td>94°C for 2 min; 94°C for 1 min; 55°C for 2 min; 72°C for 3 min</td>
<td>Bernacki et al. (1999)</td>
<td>L13283</td>
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<tr>
<td>MUC13</td>
<td>F: 5'-TGC TCC TCC CCA CAA GGA-3' R: 5'-TGG AGG CTA GTC TGC A-3'</td>
<td>73 bp</td>
<td>1 cycle: 94°C for 3 min; 35 cycles: 94°C for 1 min;</td>
<td>94°C for 3 min; 94°C for 1 min; 60°C for 1 min</td>
<td>Gipson et al. (2003)</td>
<td>AF286113</td>
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<tr>
<td>MUC15</td>
<td>F: 5'-GAA AAC AGA TGA TGG TGT GAG GAC AA-3' R: 5'-GAA AAC AGA TGA TGG TGT GAC AA-3'</td>
<td>81 bp</td>
<td>1 cycle: 94°C for 3 min; 35 cycles: 94°C for 2 min;</td>
<td>94°C for 2 min; 94°C for 2 min; 55°C for 2 min; 72°C for 3 min</td>
<td>Designed with Primer Express (Gipson) and confirmed by BLASTN and sequencing at DSCVR</td>
<td>BC020912</td>
</tr>
<tr>
<td>MUC16</td>
<td>F: 5'-GCG TCT ACC TTA AGG GTA ATG AA-3' R: 5'-GTC ACC CCA TGG CTG TGT ATS-3'</td>
<td>114 bp</td>
<td>1 cycle: 94°C for 3 min; 35 cycles: 94°C for 1 min;</td>
<td>94°C for 3 min; 94°C for 1 min; 60°C for 1 min</td>
<td>Argueso et al. (2003)</td>
<td>AF361486</td>
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<tr>
<td>MUC17</td>
<td>F: 5'-GGA CCA GCA TAG CTG CGA-3' R: 5'-GCA CTG ATG GGA ATT GTG GGA ATT GA-3'</td>
<td>91 bp</td>
<td>1 cycle: 94°C for 3 min; 35 cycles: 94°C for 1 min;</td>
<td>94°C for 3 min; 94°C for 1 min; 55°C for 2 min; 72°C for 3 min</td>
<td>Gipson et al. (2003)</td>
<td>AF430017</td>
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<tr>
<td>MUC19</td>
<td>F: 5'-ACC ACA AGT ATCCCA GCC AG-3' R: 5'-AGC TGG TGA AAG TGA GCC TA-3'</td>
<td>94 bp</td>
<td>1 cycle: 94°C for 3 min; 35 cycles: 94°C for 1 min;</td>
<td>94°C for 3 min; 94°C for 1 min; 55°C for 2 min; 72°C for 3 min</td>
<td>Chen et al. (2004)</td>
<td>AY236870</td>
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<tr>
<td>MUC20</td>
<td>F: 5'-AAC TCC AGC CCC AGC CGC CT-3' R: 5'-GGA AGC ACA CAG AGT GGT G-3'</td>
<td>360 bp</td>
<td>35 cycles: 94°C for 3 min; 35 cycles: 94°C for 1 min;</td>
<td>94°C for 3 min; 94°C for 1 min; 55°C for 30 s; 72°C for 1 min</td>
<td>Higuchi et al. (2004)</td>
<td>AB098731</td>
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<td>Androgen Receptor</td>
<td>F: 5'-GTC TCT CAA GAG TTT GGA TGG CT-3' R: 5'-GAC CTG TAC AGA GAT GAT CTG TGC-3'</td>
<td>342 bp</td>
<td>1 cycle: 94°C for 6 min; 35 cycles: 94°C for 1 min</td>
<td>94°C for 6 min; 94°C for 1 min; 55°C for 1 min</td>
<td>Lau et al. (2000)</td>
<td>M23263</td>
</tr>
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</table>

*Real-time PCR amplifications were performed over 40 cycles.
Primary antibodies used for immunohistochemistry and immunoblot analysis

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Working dilution</th>
<th>Epitope</th>
<th>Source/reference</th>
</tr>
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<tbody>
<tr>
<td>MUC1</td>
<td>HMFG-2</td>
<td>1:100</td>
<td>Tandem repeat</td>
<td>Biodesign International, Saco, ME</td>
</tr>
<tr>
<td>MUC1</td>
<td>214D4</td>
<td>1:100</td>
<td>Tandem repeat</td>
<td>J. Hilken (Wesseling et al., 1995)</td>
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<tr>
<td>MUC4</td>
<td>528</td>
<td>Undiluted</td>
<td>Peptide</td>
<td>I.K. Gipson</td>
</tr>
<tr>
<td>MUC4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1G8</td>
<td>1:100</td>
<td>ASPG-2</td>
<td>Zymed Corp., South San Francisco, CA</td>
</tr>
<tr>
<td>MUC4</td>
<td>8G7</td>
<td>1:1000</td>
<td>Tandem repeat&lt;sup&gt;c&lt;/sup&gt;</td>
<td>S.K. Batra (Moniaux et al., 2004)</td>
</tr>
<tr>
<td>MUC5AC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>791</td>
<td>1:3000</td>
<td>D3 Domain</td>
<td>Argueso et al. (Argueso et al., 2002)</td>
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<tr>
<td>MUC5AC</td>
<td>CLH2</td>
<td>Undiluted</td>
<td>Tandem repeat</td>
<td>U. Mandel (Reis et al., 1997)</td>
</tr>
<tr>
<td>MUC5B&lt;sup&gt;d&lt;/sup&gt;</td>
<td>799W</td>
<td>1:7500</td>
<td>D4 Domain</td>
<td>Gipson et al. (Gipson et al., 2001)</td>
</tr>
<tr>
<td>MUC6&lt;sup&gt;e&lt;/sup&gt;</td>
<td>CLH5</td>
<td>Undiluted</td>
<td>Tandem repeat</td>
<td>U. Mandel (Reis et al., 2000)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cryostat sections.<br> <sup>b</sup>Paraffin sections.<br> <sup>c</sup>Neuraminidase-treated paraffin sections.<br> <sup>d</sup>Antigen retrieval on paraffin sections.<br> <sup>e</sup>Independent of glycosylation/native MUC4.

**Mucin gene and protein expression profile**

Epithelia from all major regions within the male urogenital tract, from testis to foreskin, were analysed. Gene transcripts for MUC1, 3, 4, 5AC, 6, 13, 15, 17 and 20 were detected by RT–PCR (Table III, Figure 1). Human epithelia known to express each mucin gene, and their corresponding negative controls, validated all RT–PCR assays—human trachea (MUC1, 4, 5B, 7, 13, 16 and 20), small intestine (MUC2, 3 and 17), stomach (MUC5AC and 6) and salivary gland (MUC15) (data not shown). The housekeeping gene, GAPDH, was amplified in all of the samples, and only those samples with quality starting mRNA were assayed for mucin gene expression. MUC1 and MUC20 mRNAs were detected in most of the male urogenital tract epithelia, with the exception of epididymis (MUC1 only). MUC2, MUC5B, MUC7, MUC16 and MUC19 mRNAs were not detected in male urogenital tract epithelia. Of the nine mucin gene transcripts detected, well-characterized antibodies were only available for localization of MUC1, MUC4, MUC5AC and MUC6 proteins. Although MUC5B message was not detected, MUC5B protein was detected in semen; therefore, immunolocalization with antibodies to MUC5B was attempted. The results of immunolocalization of these mucin proteins in the male urogenital tract generally support the RT–PCR data, with exceptions noted below.

**Testis, epididymis and vas deferens**

Testicular tissue expressed mRNA for MUC1 and 20 in two of three samples and one of three samples expressed MUC4, 5AC and 6. The MUC1 antibodies used in this study did not bind to the testicular sections assayed, suggesting that despite the presence of MUC1 mRNA in the testis, the corresponding protein was not detected. By contrast, MUC1 mRNA (two of two samples) and protein (one of one sample) were detected in the caudal epididymis. In fact, antibodies to MUC1 bound relatively uniformly and intensely to the stereocilia on the apical membrane of the ductal epithelium (Figure 2B and C). Of all the male urogenital tract epithelia analysed, the binding intensity of MUC1 protein was the greatest in the pseudostratified epithelium of the caudal epididymis. MUC6 and MUC15 gene transcripts were also detected in one of two epididymis samples, making this the urogenital tract epithelium that expressed the fewest mucins (Table III). Only one sample of vas deferens was available for assay, and it was found to express MUC1, 3, 4, 5AC, 15, 17 and 20. Well-preserved epithelium from the vas deferens was not available for IF analysis.

**Seminal vesicle and prostate**

MUC1, MUC6 and MUC20 mRNAs were detected in five of five seminal vesicle samples, and MUC4, 15 and 17 in one or two of five samples. Three seminal vesicle sections displayed positive apical membrane binding for MUC1. Unlike the epididymis, where there was uniform binding of the MUC1 antibody, the binding pattern for MUC1 in the luminal epithelium of the seminal vesicle was patchy (Figure 3B). MUC6 protein was detected throughout the cytoplasm of some seminal

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**Table II. Primary antibodies used for immunohistochemistry and immunoblot analysis**

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<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Working dilution</th>
<th>Epitope</th>
<th>Source/reference</th>
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<td>Peptide</td>
<td>I.K. Gipson</td>
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<td>1:1000</td>
<td>Tandem repeat&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>D3 Domain</td>
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<td>U. Mandel (Reis et al., 1997)</td>
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<td>CLH5</td>
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**Table III. Results of RT–PCR for mucus on male reproductive tissue**

<table>
<thead>
<tr>
<th>MUC</th>
<th>Testis</th>
<th>Epididymis</th>
<th>Vas deferens</th>
<th>Seminal vesicle</th>
<th>Prostate</th>
<th>Bladder</th>
<th>Urethra</th>
<th>Foreskin</th>
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<td>+/(2/3)</td>
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</tbody>
</table>

MUC2, 5B, 7, 16 and 19 were not detected in any epithelium assayed. Numbers in parentheses equal PCR results/number of samples tested. A mucin gene was considered as expressed by an epithelium if any of the samples obtained showed an RT–PCR product for that gene. Scoring of PCR results by time of exposure during photography: Strong band = +/(2 s); weak band = ± (4 s); no band = −(4 s).
vesicle epithelial cells, and other cells had little, if any, antibody binding to MUC6 (Figure 3C).

Mucin gene transcripts for MUC1, 3, 4 and 20 were amplified in six of six prostate samples, and MUC5AC expression was detected in four of six prostate specimens. Regional variations in mucin protein localization, as with seminal vesicle, were noted in the prostate. Antibodies to both MUC1 and 4 bound to the apices of the epithelial cell membranes lining the lumen of the prostate gland (Figure 4B and C). MUC5AC protein was also detected in the cytoplasm towards the apical surface of the glandular epithelium (Figure 4D).

**Figure 1.** Representative RT–PCR analyses of mucin mRNA from human vas deferens, prostate and urethra. 1, MUC1 (104 bp); 2, MUC2 (441 bp); 3, MUC3 (234 bp); 4, MUC4 (101 bp); 5AC, MUC5AC (103 bp); 5B, MUC5B (405 bp); 6, MUC6 (303 bp); 7, MUC7 (901 bp); 13, MUC13 (73 bp); 15, MUC15 (81 bp); 16, MUC16 (114 bp); 17, MUC17 (91 bp); 19, MUC19 (94 bp); 20, MUC20 (360 bp); G, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (452 bp); N, no reverse transcriptase control.

**Figure 2.** Immunofluorescence (IF) localization of mucin protein in epithelial cells of the epididymis. (A) Light micrograph showing histology of epididymis tissue. (B) MUC1 protein (HMFG-2 antibody) is localized to the apical luminal surface (large arrow) of the epithelia lining the lumen of the epididymis. Nuclei (small arrows) were stained with propidium iodide to demonstrate epithelial borders. (C) Confocal microscopy image of MUC1 (HMFG-2 antibody) on the epididymis epithelium. MUC1 is specifically located on the stereocilia (large arrow) of the pseudostratified epithelium. Small arrows indicate cell nuclei. (D) Section of epididymis illustrating lack of binding of secondary antibody alone (negative control). Scale bars: A, B, D = 10 µm; C = 16 µm.

**Figure 3.** Localization of mucin protein in seminal vesicle epithelia. (A) Histology of seminal vesicle epithelium as determined by haematoxylin and eosin staining. (B) Patchy MUC1 (HMFG-2 antibody) immunolocalization to the luminal epithelium of the seminal vesicle. (C) MUC6 protein (CLH5 antibody) detected in the cytoplasm of a majority of the seminal vesicle epithelial cells. (D) Control section showing lack of non-specific binding of secondary antibody to a section of seminal vesicle. Scale bar in A = 10 µm; B, C, D are of the same magnification and scale bar = 10 µm. Intense binding illustrates positive binding for mucin protein. Nuclei of epithelia were counterstained with propidium iodide to demonstrate epithelial cells.

**Bladder, urethra and foreskin**

As determined by RT–PCR analysis, the human bladder expressed MUC1, 3, 4, 5AC, 13, 15, 17 and 20. The immunohistochemical data on MUC1, 4 and 5AC supported the RT–PCR findings. The bladder samples assayed were contracted
after fixation, as evidenced by the rounded appearance of the apical epithelial cells, the overall thickness of the transitional epithelium and the appearance of crypt-like foldings. MUC1 localized to the apical cytoplasm of the transitional epithelium at the top of the artificial folds lining the lumen of the bladder (Figure 5B). Curiously, MUC5AC antibody bound to clusters of apical cells of the epithelium, and these surface cells were filled with the mucin (Figure 5C).

Urethral epithelium expressed MUC1, 3, 4, 5AC, 13, 15, 17 and 20 (Table III). MUC1 protein distribution was prominent along the apical layer of the stratified epithelium of the urethra and, to a lesser extent, in the subapical cells of the epithelium (Figure 6B). On the contrary, MUC4 binding was more evenly distributed throughout the stratified epithelium (Figure 6C). The only secretory mucin detected in the urethra was MUC5AC, where it was localized to the glands of Littré present in the lamina propria of the urethra (Figure 6D). The foreskin, discarded following circumcision, was the most external human male urogenital tissue assayed. MUC1, MUC3, MUC4, MUC5AC, MUC13, MUC15, MUC17 and MUC20 gene transcripts were amplified, and MUC1 and MUC4 proteins (one of two samples) were detected in the foreskin (not shown), confirming the transcriptional data.

**Mucins present in seminal plasma**

Immunoblot analysis for the presence of MUC1, 4, 5AC, 5B and 6 was performed on five samples of seminal plasma from fertile men. MUC1, MUC5B and MUC6 proteins were detected, but differences in binding intensities were noted between individuals in equally loaded gels (Figure 7). The MUC1 antibody (214D4) bound to all five seminal plasma samples assayed, with two samples showing greater intensity.
Two bands were observed in one sample (lane 5) indicating polymorphism of the gene (Gendler and Spicer, 1995). MUC6 binding (CLH5 antibody) was the most variable, with MUC6 protein strongly detected in only two of five samples, with one more intense than the other. Compared with MUC1 and 6, MUC5B binding (799W antibody) was relatively uniform amongst the samples and displayed the characteristic smear observed in immunoblot detection of highly glycosylated gel-forming mucins such as MUC5B (Gipson et al., 2001). Despite the detection of MUC4 and MUC5AC mRNAs by RT–PCR in urethral tissues, these proteins were not found in the seminal plasma samples assayed (data not shown). Positive and negative controls included in the immunoblot analysis confirmed the specificity of mucin proteins detected in seminal plasma (Figure 7).

**Mucin mRNA expression in primary PrEC**

Primary PrEC cells were used for assessing the androgen regulation of mucin expression in the prostatic epithelium. DHT and mibolerone, a non-metabolizable androgen, were tested. The expression of androgen receptor and GAPDH, an internal mRNA quality control, in PrEC cells was confirmed by RT–PCR (Figure 8) before relative quantification by real-time PCR of mucin transcripts in confluent cultures.

Androgen regulation of three of the five mucins detected in the native prostatic epithelium (MUC1, 4 and 5AC) was analysed. The mRNA values of MUC1 mucin in the untreated control group served as the calibrator for the corresponding time point’s treatment groups (baseline relative expression = 1). The untreated control group expressed only MUC1 at 1, 3 and 6 days of culture (Figure 9A). MUC5AC remained undetectable throughout the time course of the experiment (data not shown). Neither DHT nor mibolerone, at any concentration, effected mucin expression. To test the PrEC cell response to dexamethasone and serum, potent regulators of mucins in ocular...
surface epithelia (Gipson et al., 2003; Hori et al., 2004), we treated PrEC cells with $10^{-6}$ M dexamethasone or switched to serum-containing media [10% (v/v)] for the duration of the experiment. Unlike ocular surface epithelia, dexamethasone had no effect on mucin gene expression, but as in ocular surface epithelia, both MUC1 and MUC4 transcripts were significantly up-regulated in the serum treatment groups. MUC1 expression peaked at 1 day post treatment, with an 18-fold increase (Figure 9A), whereas MUC4 expression peaked with a 3.3-fold increase at 6 days post treatment (Figure 9B).

Discussion

The data obtained in this study provide baseline information on mucin gene expression profiles of the male urogenital tract epithelia. The data also indicate that each region of the male urogenital tract epithelium analysed has a unique mucin gene repertoire. These variations in mucin expression may reflect specific functions of each region. The fact that testis and epididymis epithelia are most distal from the external environment (and thus the pathogen source) and have spermatozoa associated with their epithelial surfaces may be reflected in their mucin expression patterns. For example, spermatozoa are moved gently through the lumen of the epididymis epithelium by surface cilia or stereocilia that are richly invested with MUC1, which may prevent their adherence. The occlusion of the epididymis that occurs in gonorrheal and chlamydial infection (Ness et al., 1997) may be due in part to the presence of fewer mucins in this region. Similar occluding and cicatrizing pathological processes occur at the ocular surface, and in these cases, mucin-secreting cells are lost (Nelson et al., 1983; Argueso et al., 2002).

Several more membrane-associated mucins are expressed in the vas (ductus) deferens and urethra, regions in which sperm transport is rapid and driven by muscular contraction. Forces on the surface of these epithelia may be harsher, requiring better lubrication and disadhesive properties. In addition, these tubular organs are closer to the external environment than the epididymis and thus more subject to infection.

The glands emptying into the vas deferens and urethra have unique secretory products that may have caustic effects on these epithelia, thus requiring mucin protection. For example, seminal vesicle epithelium produces MUC6 that may protect its surface from its alkaline secretions. MUC6 expression is limited to the stomach and seminal vesicles, which has led Toribara et al., (1997) to hypothesize that MUC6 shields against harsh acid or alkaline environments better than other gel-forming mucins because this mucin has fewer exposed unglycosylated regions (specifically an absence of the unglycosylated carboxy-terminal D domain) that would be subject to acid/alkaline degradation. Similarly, prostatic epithelium has a large mucin gene repertoire, including several membrane-associated mucins and the gel-former MUC5AC. A large repertoire of mucins may protect this epithelium from secreted lytic enzymes. Bladder epithelium also expresses several membrane-associated mucins as well as MUC5AC, a gel-forming mucin. It too exists in a caustic, urea-rich environment, and the redundancy of expression of membrane-associated mucins, including MUC1, 3 and 4, confirming the previous findings by N’Dow et al. (2000) may indicate that a mucin-rich protective surface barrier is required by bladder epithelium.

It is not surprising that gel-forming mucins MUC5B and MUC6 are constituents of seminal plasma, because sperm motility studies indicate that cervical mucins protect sperm from the vaginal environment and assist their transport through the female reproductive tract (Eriksen et al., 1998). Mucins in the male urogenital tract may serve similar functions, especially along the urethra where both urine and sperm are conducted out of the body. Our immunoblot data detecting MUC5B protein in seminal plasma are of particular interest, because MUC5B was not detected by RT–PCR or immunohistochemistry in any of the urogenital tract epithelia analysed. Perhaps the Cowper’s glands, which were not available for study, could account for this discrepancy. However, only 5% of seminal secretions originate from these secondary glands; prostate and seminal vesicle secretions account for the majority of the seminal volume (Owen and Katz, 2005). Unlike MUC5B, MUC4 and MUC5AC proteins were not detected in the seminal plasma samples despite finding their transcripts in the male urogenital epithelia, especially in the prostate and urethra. Although it is unclear why these mucin proteins were not found, proteases, such as trypsin and prostate specific antigen, have been found in seminal fluid (Paju et al., 2000) and may destroy the antigen-binding sites on these mucins.

By comparison with the endocervical epithelium of the female reproductive tract, where three of the large gel-forming mucins, MUC5AC, 5B and 6, were detected in some epithelial cells (Gipson et al., 1997), no such protective redundancy was identified in any cells of male urogenital tract epithelia examined. With the exception of seminal vesicle and epididymis epithelia, MUC5AC was detected in all epithelia assayed, whereas seminal vesicle expressed only MUC6 (mRNA and protein). Our data corroborate previous findings, regarding seminal vesicle epithelial expression of MUC6 (Ho et al., 1995; Bartman et al., 1998; Leroy et al., 2003).

MUC2 and MUC6 expressions in the prostate are controversial. Durrant et al. (1994) detected MUC2 by immunohistochemistry, using the monoclonal antibody 996/1, whereas Ho et al. (1993) and Gold et al. (1994) failed to identify MUC2 expression in the prostate by Northern blot and immunostaining techniques. A similar dichotomy exists for MUC6 reports—detected by Northern blot and immunostaining (Ho et al., 1993; Gold et al., 1994) but not by ISH or immunohistochemistry (Bartman et al., 1998; Leroy et al., 2003). Our data indicate that neither MUC2 nor MUC6 is present in prostatic epithelium.

The mucins in the human female reproductive tract are hormonally regulated. Cervical MUC4 and MUC5B expressions peak when progesterone levels are low (before midcycle) in normally cycling women (Gipson et al., 1999), and the highest levels of MUC5B protein are detected in cervical mucus at midcycle (Gipson et al., 2001). Unlike female reproductive tract mucins, our data show that prostatic epithelial mucins MUC1 and 4 do not appear to be under hormonal control. O’Connor et al. (2005) reported similar findings for MUC1 when PrEC cells were cultured with DHT.
To our knowledge, the data reported in this study provide the most inclusive mucin profile of the human male urogenital tract epithelia to date. Even so, inherent limitations of this study exist. Because the samples consisted of discarded surgical and post-mortem epithelia, the quantity of usable specimens collected varied from one to nine individuals per epithelial type. Moreover, epithelia from all areas of interest within the male urogenital tract were not available for collection. Efferent ducts, ejaculatory ducts and Cowper’s gland, a potential major mucin source, were not available for study.

Further study of mucins in the male urogenital tract may yield information regarding the functional importance of specific mucins in the maintenance of normal reproductive health, including defense against pathogens. The mucin profile and expression level in male urogenital tract epithelia in diseased states, such as infections with sexually transmitted pathogens, will thus be of interest, particularly if down-regulation occurs. Effects of inflammation resulting from infections on mucin expression have not been investigated in these epithelia, and, because information on regulators of mucin expression is beginning to be known, therapeutic agents targeted at regulation may be feasible.

In conclusion, tissue-specific mucin repertoires were observed in the human male urogenital tract epithelia; MUC1 and MUC4 expressions in prostatic epithelium are not under direct androgenic control.

Acknowledgements

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References


Mucins in the male urogenital tract


