The expression of MUC1 in human Fallopian tube during the menstrual cycle and in ectopic pregnancy

M. Al-Azemi, B. Refaat, J. Aplin, and W. Ledger

1Academic Unit of Reproductive and Developmental Medicine, Level 4, The Jessop Wing, Royal Hallamshire Hospital, Sheffield S10 2SF, UK
2Medical School and School Biological Sciences, University of Manchester, Manchester, UK
3Correspondence address. Tel: +44-0114-2268317; E-mail: alazemimajda@hsc.edu.kw

BACKGROUND: Ectopic pregnancy is a major cause of maternal morbidity and mortality with increasing incidence worldwide. We have investigated whether epithelia from Fallopian tubes (FTs) bearing an ectopic pregnancy differ from normal tubes in expression of MUC1.

METHODS: Since it is not possible to collect FTs from women carrying a healthy pregnancy, we studied tissue collected at the time of hysterectomy for benign disease. Women were injected with hCG in the days leading up to hysterectomy, and pseudopregnancy confirmed by the presence of high serum progesterone levels and the decidualization of the endometrium. FTs from the different stages of the menstrual cycle (n = 24), tubes bearing an ectopic pregnancy (n = 15) and pseudo-pregnant tubes (n = 6) were collected and examined using immunohistochemistry and quantitative RT–PCR.

RESULTS: MUC1 was present at the apical surface of the tubal epithelial cells throughout the menstrual cycle, but intracellular localization was minimal in the follicular phase, increasing to a maximum in the luteal phase. MUC1, including the glycoform recognized by antibody 214D4, was found at the apical surface of tubal epithelium in both the ectopic and pseudo-pregnant groups and the intracellular expression was much stronger in the pseudo-pregnant group than in the ectopic group. The 214D4 epitope was absent from tubal tissue adjacent to ectopic implants.

CONCLUSIONS: The decrease in MUC1 expression and altered glycosylation in tubal epithelium from ectopic pregnancy may reflect an increase in receptivity.

Key words: Fallopian tube / ectopic pregnancy / MUC1 / progesterone receptors

Introduction

Mucins are high molecular weight glycoproteins that provide a protective layer on epithelial surfaces. Nineteen human mucins have so far been identified (Andrianifahanana et al., 2006). MUC1 is a heavily glycosylated type-I transmembrane mucin that is expressed on the apical surface of upper reproductive tract epithelial cells (Meseguer et al., 2001). MUC1 protein is composed of short cytoplasmic and transmembrane domains and a large extracellular domain. The extracellular domain in humans contains 20–125 tandem repeats of 20 amino acids that are rich in serine, threonine and proline residues and prone to extensive O-glycosylation (Meseguer et al., 2001; Brayman et al., 2004). The ectodomain is estimated to project 200–500 nm above the cell surface. Its high degree of glycosylation provides lubrication, prevents dehydration and offers protection from proteolysis (Brayman et al., 2004). MUC1 also possesses anti-adhesive properties and modulates cell–cell and cell–extracellular matrix interactions by steric hindrance (Aplin et al., 2001; Meseguer et al., 2001; Brayman et al., 2004) due to the high surface density and the large, extended ectodomain.

MUC1 has been suggested to act as a barrier to embryo implantation in a number of animal species (Aplin et al., 2001; Meseguer et al., 2001; Brayman et al., 2004). MUC1 is lost both in mice (Braga et al., 1993; Surveyor et al., 1995) and rats (DeSouza et al., 1998) throughout the uterine epithelium during the implantation phase. MUC1 is also absent in pig endometrium during the implantation window (Bowen et al., 1996) and has been reported to decline in the baboon endometrium during the receptive phase (Hild-Petito et al., 1996). On the other hand, MUC1 is up-regulated during the receptive phase in rabbit but it is locally removed at specific implantation sites (Hoffman et al., 1998). Human MUC1 is also up-regulated in luminal and glandular endometrial epithelial cells during the implantation phase (Hey et al., 1994; Meseguer et al., 2001) and in vitro studies suggest that the human blastocyst produces...
Factors that induce local removal of MUC1 to facilitate implantation on monolayers of human uterine epithelium (Meseguer et al., 2001).

The expression of MUC1 is progesterone dependent (Hey et al., 1994; Aplin et al., 1998) and in vivo studies have shown that MUC1 is up-regulated in human endometrial epithelial cells during the luteal phase (Meseguer et al., 2001). In vitro data from human endometrial, breast and prostate carcinoma cell lines suggest that estrogen alone has no effect on the expression of MUC1 mRNA or protein (Gollub et al., 1995; Botti et al., 1997; McGuckin et al., 1998; Mitchell et al., 2002). However, estrogen followed by progesterone, or progestesterone alone, stimulates MUC1 mRNA and protein expression in some prostatic and breast carcinoma cell lines (Gollub et al., 1995; Botti et al., 1997; McGuckin et al., 1998; Mitchell et al., 2002).

Uniquely in the animal kingdom, humans are susceptible to ectopic pregnancy (EP), which usually occurs in the Fallopian tube. EP is associated with significant maternal morbidity and mortality, and its incidence is increasing worldwide (Kriebs and Fahney, 2006). EP is the leading cause of pregnancy-related death in the first trimester, accounting for 9% of all pregnancy-related deaths (Lewis, 2001).

MUC1 expression in human endometrium has been investigated using antibodies that recognize peptide epitopes within the tandem repeats (Hey et al., 1994, 1995). Antibodies including HMFG1, HMFG2, BC2, BC3 and 214D4, vary in their ability to detect glycoforms of the mucin, which show time and region-specific expression (Aplin et al., 1998; DeLoia et al., 1998). Such structural differences may affect the function of MUC1 in physiological and pathological states. MUC1 is expressed by human Fallopian tube epithelial cells (Hey et al., 2003) and may be a key factor in preventing embryo implantation and the development of EP. In the present study, we have used two monoclonal MUC1 antibodies, BC2 and 214D4, to detect MUC1 core protein and a hormonally regulated glycoform during the menstrual cycle in human Fallopian tube, and to determine whether abnormal tubes bearing an EP differ from healthy tubes in the expression and glycosylation of MUC1.

Materials and Methods

The study was approved by South Sheffield Ethics Committee and informed written consent was obtained prior to the collection of tissue samples. All specimens were collected at the Jessop Wing, Royal Hallamshire Hospital in Sheffield, UK.

Menstrual cycle group

Twenty four Fallopian tubes were collected at the different phases of the menstrual cycle from 12 cyclic women (median age 34 years, range 28–40 years) who were undergoing routine total abdominal hysterectomy (TAH) for benign disease not affecting the Fallopian tubes. All women had regular menstrual cycles, were of proven fertility with no evidence of tubal disease and were not taking exogenous hormones.

Histological dating was carried out according to the criteria of Noyes et al. (1975) to confirm the phase of the menstrual cycle and all endometrial samples from the different phases of the cycle were consistent for the patient’s reported last menstrual period (LMP), steroid hormones concentrations and histological stage (Table I).

EP group

Fifteen Fallopian tubes were collected from women (median age 29 years, range 24–36 years) diagnosed with EP for whom salpingectomy was performed on clinical management grounds. Mean gestational age calculated from the LMP was 6.5 ± 1.5 week and human chorionic gonadotrophin (hCG) concentrations were 3480.28 ± 620.64 IU/l. All participants in this group conceived spontaneously and were not taking exogenous progesterone.

‘Pseudopregnancy’ group

Since it is not ethically possible to collect Fallopian tubal tissue from women carrying a healthy pregnancy, we studied tissue collected at the time of hysterectomy. Women were asked to have treatment with hCG in the days leading to hysterectomy. This regime produces a state of ‘pseudopregnancy’, is harmless and has been previously used within research studies (Refaat et al., 2008).

Six Fallopian tubes were obtained from three women (median age 38 years, range 36–41 years) who were undergoing routine TAH for benign disease not affecting the Fallopian tubes. All women had regular menstrual cycles and were of proven fertility with no evidence of tubal disease. These patients were pre-treated with subcutaneous hCG (5000 IU) every 3 days beginning in the mid-luteal phase for at least 12 days prior to TAH. The three included patients had had 9, 6 and 10 doses, respectively.

An endometrial biopsy and a blood sample were collected at the time of hysterectomy to examine endometrial decidualization and hCG concentrations. The state of pseudopregnancy was confirmed by the persistence of amennorhea, hCG concentrations, and the corpus luteum being identified on the day of operation. The menses were delayed in the patients by 24, 15 and 30 days, respectively, and hCG concentrations on day of salpingectomy were 124.66 ± 44.80. Histological criteria of early pregnancy such as glandular secretion, decidualization of stromal fibroblasts and appearance of uterine natural killer cells were also observed in all endometrial samples.

Sampling and processing

For the case group, the Fallopian tubes were excised at least one centimetre away from the implantation site to avoid collecting any embryonic or trophoblastic tissue and to assure the integrity of tubal morphology and function. The ampullary and isthmic regions of the excised tubes from the three groups were identified and a small section immediately cut from each region using RNAase-free equipment (baked at 200°C for 4 h). These samples were then divided into three equal pieces, with one piece being immediately fixed in 10% buffered formalin for immunohistochemistry and the other parts in 5 ml of RNAlater solution (Ambion, UK) for the RT–PCR. All the tissues used in the RT–PCR were snap-frozen in RNAlater solution (Ambion, UK) and stored at −80°C until fixed in 10% buffered formalin to preserve the RNA stability.

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Table I Summary of steroid hormones concentrations and histological staging of the menstrual cycle (12 patients)

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>E2 (pmol/l)</th>
<th>P (pmol/l)</th>
<th>Histological dating</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>227.25 ± 65.34</td>
<td>2.97 ± 0.67</td>
<td>Menstrual phase</td>
</tr>
<tr>
<td>4</td>
<td>455.75 ± 237.24</td>
<td>7.27 ± 2.62</td>
<td>Follicular phase</td>
</tr>
<tr>
<td>4</td>
<td>335.00 ± 63.23</td>
<td>27.10 ± 6.80</td>
<td>Secretory phase</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. E2, estradiol; P, progesterone.
Antibodies
Two monoclonal antibodies directed to MUC1 ectodomain, 214D4 and BC2 were used in this study. Monoclonal mouse anti-human MUC1 breast cancer (BC)-2 (IgG) antibody was obtained from Serotec (Oxford, UK) although MUC1 monoclonal antibody 214D4 was kindly provided by Professor John Hillens. Mouse human-PR monoclonal antibody (AB-52; Santa Cruz, Santa Barbara, USA) which recognizes both PR-A and PR-B was used.

Immunohistochemistry
An avidin–biotin horseradish peroxidase technique was used to localize the expression of MUC1 in the different samples using both MUC1 antibodies. Briefly, sections were dewaxed, dehydrated in alcohol, and treated with 2% (vol/vol) hydrogen peroxide for 20 min in methanol to block endogenous peroxidase. The sections were incubated for 30 min with normal horse serum (Vector Laboratories, Burlingam, CA, USA) and then incubated with the primary antibodies overnight at 4 °C. The antibody concentrations were 1:1000 and 1:20 for BC2 and 214D4, respectively. The following day the sections were washed with 20 mM phosphate buffered saline (pH 7.3) and then incubated with 1:200 biotinylated horse anti-mouse secondary antibody for 30 min. After a further wash step, the sections were incubated with the avidin–biotin peroxidase complex ELITE system (Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min and then subsequently with 3,3′-diaminobenzidine (Vector Laboratories, Inc., Burlingame, CA, USA) for 10 min. Sections were washed in tap water, counterstained with Gill’s haematoxylin, then dehydrated in a series of graded ethanol, cleared in xylene and mounted in DPX (BDH/Merck).

Sections from the endometrial samples collected from the menstrual cycle groups were used as positive controls, as endometrium is known to express the antigen of interest (Hey et al., 1994; Wang et al., 2008). The negative control slide consisted of a section of the tissue blocks being studied and it was treated identically to all other slides, with the exception that the primary antibody was omitted to control non-specific binding of the detection system.

The sections were observed on a Labor Lux microscope (Leitz, Wetzlar, Germany), at a magnification of ×100, ×250 and ×400. A positive reaction for MUC1 expression was characterized by the presence of brown staining. Each section was examined by two observers who were blinded to the source of tissue and they agreed on the intensity of staining according to the following semi-quantitative scale: (−), negative; (+), equivocally positive; (++), weakly positive; (+++), positive; (++++) strongly positive. Representative sections were photographed using an Olympus digital camera at ×250 and ×400 magnification.

Progesterone receptor immunopositivity was semi-quantitatively evaluated. Reactions were interpreted as positive based on nuclear staining. Immunohistochemical staining results were scored based on the percentage of cells showing expression. The scoring system was: 0 = ≤5%; +1 = 6–25%; +2 = 26–50%; +3 = 51–75%; +4 = 76–100%. The intensity of staining was not used for scoring as the nuclear staining was fairly homogeneous.

Laser capture microscopy
For investigation of mRNA expression levels, epithelial cells from the different segments of the Fallopian tube were microdissected using a laser capture microdissector (Arcturus, Herfordshire, UK) as previously described (Refaat et al., 2008). Briefly, tissues were moved from −80 °C, fixed in 10% formalin and embedded in paraffin 24 h prior to sectioning. Sections of 10-μm thickness from the Fallopian tube were dewaxed, dehydrated in alcohol, and then stained with toluidine blue (1 min) and destained and dehydrated through graded ethanol concentrations and two 5-min xylene washes.

cDNA synthesis
RNA was extracted from Fallopian tubes using the RNeasy FFPE Kit (QIAGEN Ltd, Crawley, UK) following the manufacturer’s instructions. RNA was treated with RNase-free DNAse during the extraction protocol. The First strand complementary DNA (cDNA) was carried out using the RETROscript kit (Applied Biosystems, Warrington, UK) and by following the manufacturer’s protocol. Briefly, reverse transcription (RT) was performed with heat denaturation of the RNA (75 °C, 3 min) by adding 2 ng total RNA from each group under investigation and using random decamers in the presence of RNase inhibitor (50 °C, 1 h).

Primers to identify a MUC1 transcript
The complete mRNA sequence of human MUC1 (GenBank AY463543) was used to design a primer pair for identification of MUC1 transcript. The forward primer begins at base 3869 in exon 6 and the reverse one begins at base 4021 in exon 7 (Table II).

Quantitative PCR
Polymerase chain reaction (PCR) was performed with the cDNAs, power SYBR green master mix (Applied Biosystems, Warrington, UK), and primers from Metabion, Germany (Table II). Each well of the PCR plate contained 10 μl SYBR Green, 7 μl water, 1 μl of each primer (20 pmol) and 1 μl cDNA. The amplification was performed under the following conditions: 50 cycles (95 °C 30 s, 65 °C 30 s, 72 °C 30 s). Universal human RNA (Stratagene, Amsterdam, Netherlands) was used as a positive control and two negative controls were included, one with minus-reverse transcription (minus-RT) control from the previous step and a minus-template PCR, which contained all the PCR components but water was used as a template. All experiments were performed in triplicate.

Results were analysed using Mx3000P (Stratagene, Hemel Hempstead, UK). Relative MUC-1 expression quantities were compared between the ectopic and pseudo-pregnant (SP) groups, and between the different phases of the menstrual cycle. The threshold cycle values were normalized against threshold value of human β-actin and data was analysed using the formula 2−ΔΔCT. The results were expressed as mean ± SEM. Statistical analysis was performed by using Student’s t-test to compare the SP and EP groups, although one way ANOVA was used to examine the expression of MUC1 mRNA in the menstrual and luteal phases compared with the follicular phase during the menstrual cycle. P < 0.05 was considered significant.

Table II Showing sequences of PCR primers used for detection of β-actin and MUC1

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>5‘ATCCCCCAAGTTCAACATG 3’</td>
<td>5‘GTGGCTTTAGGATGGCAAG 3’</td>
</tr>
<tr>
<td>MUC1</td>
<td>5‘TCGCTTGCTGCTGTCAGT 3’</td>
<td>5‘TAGGTATCCGGGCTGGAA 3’</td>
</tr>
</tbody>
</table>
Results

Immunohistochemistry

Menstrual cycle group

The two anti-MUC1 antibodies were tested against endometrium at various cycle phases. A positive reaction was characterized by the presence of brown staining in sections from endometrial biopsies collected from the menstrual cycle groups as previously verified (Hey et al., 1994; Wang et al., 2008), with staining confined to the epithelium in all cases (data not shown). BC2, which recognizes an epitope on the core protein, was found to stain much more strongly in luteal than in follicular phase. 214D4, which recognizes a putative glycan epitope added late in the secretory pathway, was found at epithelial apical surfaces, and intracellularly only in the secretory phase. Negative controls, created by omitting the primary antibody, failed to show any positive reaction (data not shown).

In tubal tissue, the two antibodies produced similar immunoreactivity in both the ampulla and isthmus in all samples (data not shown). The apical surfaces of the tubal epithelial cells were clearly labelled during the different stages of the menstrual cycle. However, intracellular localization of MUC1 was minimal or almost absent in the follicular phase (Fig. 1A and B), increasing to a maximum during the luteal phase (Fig. 1D and E) and then decreasing in the menstrual phase (Fig. 1G and H) but remaining at levels higher than the follicular phase (Table III). The two epitopes showed differences in intracellular distributions that were most clearly evident in the follicular phase. BC2 localized basally beneath the epithelial nucleus (Fig. 1A), although 214D4 (Fig. 1B) was seen in the apical cytoplasm where, in endometrial epithelium as well as tubal epithelium, Golgi-associated and secretory compartments are found (Dockery et al., 1988; Crow et al., 1994). The intensity of intracellular immunoreactivity was less with 214D4 (Fig. 1B, E and H) than with BC2 (Fig. 1A, D and G).

Progesterone A and B nuclear receptors demonstrated immunoreactivity during all menstrual cycle phases. The staining score (expressed as mean ± SD) was highest during follicular (87.57 ± 9.4), decreased in luteal phase (73.94 ± 9.8) and was at its lowest during the menstrual phase (45.40 ± 10.8) (P < 0.05) (Fig. 1).

Ectopic and pseudopregnancy groups

Using BC-2 and 214D4 antibodies, MUC-1 was localized at the apical surface of tubal epithelium in both the ectopic and SP groups (Fig. 1I and K for EP; M and N for pseudopregnancy). Intracellular MUC1 was higher in the SP group compared with the ectopic group as detected by antibody BC2. Antibody 214D4 showed no intracellular reactivity in the latter group (Fig. 1K and N and Table III). Occasional gaps in apical cell surface staining a few cells in diameter were observed in the ectopic group.

Antibody against progesterone A and B nuclear receptors demonstrated immunoreactivity in both the ectopic and SP groups (Fig. 1L and O, respectively). The antibody clearly stained some of the nuclei of the tubal epithelial and stromal cells in both groups. Receptor immunolocalization (expressed as mean ± SD) was significantly greater in the SP group (85.50 ± 10.04 versus 64.2 ± 12.76; P = 0.017).

Quantitative RT–PCR

Quantitative real-time PCR experiments were carried out on all Fallopian tube specimens collected from the different groups. β-actin mRNA showed no statistically significant variation between the different samples (P > 0.05). MUC-1 mRNA was lowest in the follicular phase, increased by almost 10-fold in the luteal stage and then decreased in the menstrual stage but remained higher than the follicular stage (P < 0.05) (Fig. 2A). MUC-1 mRNA was significantly higher in the SP group than in tubes from EP (P < 0.05) (Fig. 2B).

Discussion

We here report cyclicity in MUC1 expression in tubal epithelial cells with highest expression in the luteal phase and lowest in the follicular phase. In human Fallopian tubes bearing EP a decrease is seen in MUC1 mRNA and protein compared with pseudopregnancy. It has been suggested that MUC1 acts as a barrier, inhibiting premature embryo attachment at an anatomically inappropriate site such as
Table III  Summary of the intensity of immunohistochemical expression of MUC1 protein in human Fallopian tube using the BC2 and 214D4 antibodies during the different phases of the menstrual cycle, pseudopregnancy and EP

<table>
<thead>
<tr>
<th>Menstrual Cycle</th>
<th>Apical localization</th>
<th>Intracellular localization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BC2</td>
<td>214D4</td>
</tr>
<tr>
<td>Follicular</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Luteal</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Menstrual</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Pseudopregnancy</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>EP</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

Figure 2  Quantitative RT–PCR analysis of MUC1 mRNA in human Fallopian tubes.
(A) MUC1 mRNA expression in Fallopian tubes collected during the different phases of the menstrual cycle (*P < 0.05). (B) MUC1 mRNA expression in Fallopian tubes collected from EP and SP groups (*P < 0.05).

The expression of MUC1 is regulated by sex steroid hormones in a variety of tissues (Braga et al., 1993; Surveyor et al., 1995; Meseguer et al., 2001). Emerging data from in vivo and in vitro studies suggest that MUC1 expression is regulated by progesterone both in endometrium (Hey et al., 1994; Aplin et al., 1998; DeLoia et al., 1998, Meseguer et al., 2001; Horne et al., 2006) and in prostatic and breast carcinoma cell lines (Gollub et al., 1995; Botti et al., 1997; McGuckin et al., 1998, Mitchell et al., 2002). Here we confirm previous reports (Land and Arends, 1992; Sadan et al., 2002) of low abundance of progesterone receptor in tubal tissue from EP, and show increased abundance in pseudopregnancy. However, there is evidence to support the involvement of non-steroidal signals in MUC1 regulation, some of which may be paracrine in origin (Bazer and Slayden, 2008); both tumour necrosis factor-alpha and interferon-gamma increase MUC1 synthesis, although the Protein Inhibitor of Activated Signal protein family represents a potential source of factors in the uterus that can dramatically repress basal MUC1 promoter activity. This repression is not wholly overcome by treatment with progesterone (Bryman et al., 2007).

The observed differences in MUC1 gene and protein expression between the ectopic and SP groups may be due to embryonic signaling to maternal tissues, but at present remain unexplained. Discrepancies in the duration of amenorrhoea and the difference in hCG concentrations should be kept in mind. The period of the pregnancy in all ectopic cases was longer than the period of the state of pseudopregnancy that we were able to establish with repeated hCG injection. We believe that it would have been unethical to prolong the SP state and consequently delay elective surgery for more than 21 days. Recruitment to the SP arm was also difficult and therefore we present control data on only six tubes, although all findings were consistent between tubes and patients. Therefore the observed differences in protein and gene expression could be related to the length of time that the tissues were exposed to hCG rather to a difference between ‘healthy’ and ‘pathological’ tubes.

Tubal pregnancy has long been attributed to failure of the tubal transport mechanism due to impaired rhythmic smooth muscle contraction and ciliary beat activity. The decrease in MUC1 expression in EP indicates an alteration in tubal epithelial properties in women...
suffering EP. Such changes may impact on both the receptivity of the tubal surface towards a potentially implanting embryo and the ability of the tubal environment to facilitate zygote transport to the uterus. Further studies are needed to explore this hypothesis.

References


Wang P, Julian JA, Carson DD. The MUC1 HMF1 glycoform is a precursor to the 214D4 glycoform in the human uterine epithelial cell line, HES. *Biol Reprod* 2008;78:290–298.

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