Introduction

It is generally accepted that the process of implantation that begins with intimate association of the conceptus and uterine endometrium, ending with the formation of a placenta, involves an adhesion cascade. Adhesive uterine luminal epithelium (LE) ligands, normally masked by mucins, become exposed during the receptive period, and then various adhesion molecules act sequentially, or in parallel, to stabilize adhesion of the conceptus trophectoderm to LE (Kimber, 2000; Pellicer et al., 2002).

The cell surface mucin MUC1 is expressed in endometrial epithelial cells with increased abundance in the secretory phase of the menstrual cycle. The presence of this maternal cell surface glycoprotein acts as a barrier to embryo implantation, arising from the anti-adhesive property of MUC1, maintaining a non-receptive uterine state (Hey et al., 2003). Further study has shown that the human blastocyst was capable of decreasing endometrial MUC1 expression (Meseguer et al., 2001; Pellicer et al., 2002). Similarly in sheep, an immunoreactive MUC1 was highly expressed at the apical surfaces of uterine luminal (LE) and glandular epithelium (GE) in both cycling and pregnant ewes, but was decreased dramatically and nearly undetectable on LE when intimate

Abstract

The transmembrane mucin glycoprotein (MUC1) has an anti-adhesive role, and functions to maintain a non-receptive uterine state. A polymorphic variation of the MUC1 gene has been associated with female infertility due to suspected failure of embryo implantation, based on the significant greater size of the lower allele observed in infertile women. The aim of this study was to confirm this preliminary observation using long polymerase chain reaction (PCR), which has amplified the 60-bp polymorphic variable number of tandem repeat (VNTR) associated to the binding domain of the MUC1 glycoprotein. DNA samples were obtained from 20 women, 10 fertile and 10 infertile, and the VNTR region was amplified through a long PCR procedure. The VNTR size range from 1.6 to 2.9 kb (22–44 motifs). The average size for the lower allele was 1.69 kb for both groups, and for the upper allele was 2.35 and 2.49 kb (P > 0.05) for fertile and infertile groups respectively. The VNTR polymorphism of the MUC1 gene was not associated with female infertility, although its significance cannot be discarded. It is suggested that other regulatory molecules and signals may interact with the MUC1 gene variations, favouring endometrial receptivity and embryo attachment.

Keywords: embryo, implantation failure, infertility, MUC1, VNTR
contact between LE and trophectoderm began (Johnson et al., 2001). All these studies clearly show that in humans, as in sheep, rodents and pigs (Bowen et al., 1996), the human adhesion cascade initiates through down-regulation of MUC1.

Mucin 1 (MUC1) is a transmembrane glycoprotein greater than 300 kDa, heavily O-glycosylated at the cell surface, which may be secreted by release from intracellular granules or by dissociation of cell surface associated moieties (Parry et al., 2001). MUC1 acts to lubricate the epithelial cell surface and protecting the epithelium from microbial invasion. It may also play a significant role in cell–cell interactions, metastasis, signalling, and other biological processes (Wesseling et al., 1995). MUC1 is a type 1 intercalated plasma membrane molecule with a large extracellular domain and a short cytoplasmic sequence. The extracellular domain contains a variable number tandem repeat (VNTR) sequence of 20 amino acids. The number of repeats varies from about 20 to 80 in the normal population, and individuals carry two codominantly expressed alleles. As a result, the core protein varies in the range 120–220 kDa, rising to over 400 kDa with glycosylation (Gendler et al., 1990; Lancaster et al., 1990). The MUC1 gene comprises 7 exons and varies in size from 4 to approximately 7 kb, depending on the number of tandem repeats in exon 2 (Gendler et al., 1990; Lancaster et al., 1990). Although most of the gene size variation is linked to the number of VNTR, a polymorphism within the exon 2 has also been reported, a G/A substitution, which is responsible for a splicing variation of the MUC1 transcript that could lead to a different protein conformation (Ligtenberg et al., 1990, 1991). Variations on the protein level have also been demonstrated by the occurrence of substitutions in the VNTR domain at three different positions, and these sequence variations may induce important consequences to conformation (Engelmann et al., 2001). This domain presents an immunodominant epitope, a short peptide motif (DTR) within the mucin, which accounts for a concerted replacement of residues Asp–Thr to Glu–Ser in more than 50% of the repeated units. Other types of MUC1 variations may arise from alternative splicing. A splicing variant that lacks the transmembrane and cytoplasmic sequences as well as the proteolytic cleavage site presents a truncated form of the MUC1, named MUC1/SEC (Wreschner et al., 1990; Weiss et al., 1996). However, based on immunological studies, both MUC1 and MUC1/SEC are located on the apical surface of the tubal epithelium and may contribute to the anti-adhesive character of the tubal surface, inhibiting ectopic implantation (Hey, 2001). MUC1 acts to lubricate the epithelial cell surface and protecting the epithelium from microbial invasion. It may also play a significant role in cell–cell interactions, metastasis, signalling, and other biological processes (Wesseling et al., 1995). 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Considering the VNTR size polymorphism, it has been proposed that women with unexplained infertility might have a genetic susceptibility to failure of embryo implantation due to a smaller MUC1 allele size (Horne et al., 2001).

The objectives of this investigation were to determine and compare the allele sizes of the MUC1 60-bp VNTR through long polymerase chain reaction (PCR) and PCR–restriction fragment length polymorphism (RFLP) techniques in fertile and fertile women and to discuss the molecular mechanisms involved in the embryo implantation failure, focusing on the highly polymorphic MUC1 gene.

Materials and methods

Patients from the Human Reproduction Centre (CRH, Ribeirao Preto, Brazil) were investigated with regard to the association between MUC1 VNTR polymorphism and embryo implantation failure. This research was under ethical clearance and approval and all patients signed a consent form. Blood samples were obtained from 10 infertile women with three or more implantation failures, and the control group consisted of 10 fertile women with two or more successful pregnancies. DNA was extracted organically from peripheral blood samples of the 20 unrelated women.

Genomic DNA used as the template was obtained from blood samples by adding 1 ml of lysis buffer (20 mmol/l Tris–HCl pH 7.5, 5 mmol/l ethylenediaminetetraacetic acid–EDTA pH 7.5, 640 mmol/l sucrose, 10 mmol/l MgCl2, 4% Triton X-100) to 500 µl of leukocytes, kept on ice for 10 min. Lysed cells (white pellet) were precipitated by centrifugation at 7200 g for 1 min at 4°C, followed by two or more washes with lysis buffer diluted 1:1 v/v in water. Thus, the pellet was submitted to digestion with 10 µl of proteinase K (10 mg/ml) and 200 µl PK buffer (10 mmol/l Tris–HCl pH 7.5, 1 mmol/l EDTA pH 7.5 and 1% sarkosyl), which was incubated overnight at 50°C. After incubation, 500 µl of 8 mol/l guanidine isothiocyanate/0.49 mol/l ammonium acetate was added to the digestion solution and shaken for 1 h at room temperature to dissolve the pellet. The DNA was precipitated with 800 µl of 100% isopropanol, centrifuged at 7200 g for 10 min, and the supernatant was discarded. Two more washes were carried on with 60% isopropanol and centrifuged at 7200 g for 2 min. The DNA was dried and redissolved in 0.2 ml of TE (10 mmol/l Tris–HCl pH 7.5 and 1 mmol/l EDTA), which was incubated at 65°C for 1 h for complete dilution.

DNA samples were amplified in the following PCR conditions: 20 pmol of primers, 1.0 mmol/l MgSO4, 300 µmol/l of dNTPs, 2.5 IU of PfX DNA polymerase (Invitrogen Inc.), 100 ng of DNA template, 1 × PfX buffer, and 1 × enhancer buffer (Invitrogen Inc.). Primers were designed to flank the 60-bp VNTR region as described in Figure 1A.

A PCR–RFLP system was utilized to determine if the amplified products were not PCR artefacts. Amplicons were digested with DdeI restriction endonuclease, the same enzyme used in Southern Blots to characterize MUC1 VNTR by Horne et al. (2001), and the number of motifs was calculated based on its sequence expected size (Figure 1B).

PCR amplification consisted of the following condition: 98°C for 10 min, 68°C for 10 min, 94°C for 3 min, followed by 9 cycles of 94°C/35 s, 63°C/1 min, 68°C/2.5 min, and 25 cycles of 94°C/35 s, 65°C/1 min, 68°C/2.5 min with a final extension of 10 min at 68°C.

The estimated base pair sizing of the amplicons (Figure 2) was performed based on a DNA sizing ladder (1 kb ladder; Invitrogen Corporation, CA, USA) detected in a video documentation system (ImageMaster VDS System–IMVDS; Pharmacia Biosciences, San Francisco, CA, USA).
Figure 1. MUC1 VNTR genotyping through PCR–RFLP. (A) MUC1 DNA sequence of the amplified region. The black arrows within the sequence indicate the DdeI restriction site. (B) PCR–RFLP schematic view of a hypothetic amplicon and its restriction map. Number of VNTR repeats is calculated by number of VNTR motifs = X – 65/60 bP = Y repeats, where X is the largest observed fragment size, e.g. VNTR no. = 305 – 65/60 = 4 repeats.

Figure 2. MUC1 VNTR polymorphism (exon 2) of infertile and fertile women detected in 1% agarose gel electrophoresis. Lane M, 1 kb DNA ladder; lane N, negative control; lanes 1, 3, 5, 7, 9, 11, 13, 14 are from infertile women; lanes 2, 4, 6, 8, 10, 12 are from fertile women.
VNTR genotypes of both groups were statistically compared by analysing the number of repeats and the allele size averages through t-test.

Results

The low resolution of agarose gel electrophoresis for detection of larger fragments cannot clearly distinguish closer alleles above 1.5 kb; therefore, allele size estimates were performed through video documentation system software, which captured and processed all the images, estimating DNA migration distortion and calibrating the average size for each sample. Bands were calculated through a regression curve based on the DNA size ladder migration in both sides of the gel. Distorted bands were corrected by the IMVDS software, using the DNA size ladder as calibrator of the gel.

Allele sizes and number of motifs for all patients are described in Table 1. The estimates were based on DNA migration (Figure 2). Allele sizes were quite similar, with both groups having the same average size for the lower allele (1.69 kb). For the upper allele, infertile women had an average of 2.49 kb, compared with 2.35 kb for fertile ones. The 60-bp VNTR polymorphism of the MUC1 gene was not significantly different between the two groups ($P > 0.05$). Four patients, two infertile and two fertile, had no amplification, although their $\beta_2$-microglobulin (positive control) showed normal amplification, probably due to very large alleles or to their complete absence.

The various faint bands seen at the bottom of the agarose gel electrophoresis (Figure 2) represent a ladder of amplicons originating from abnormal amplification of the repeated motifs during PCR reactions, usually seen when DNA sequence shows a higher GC content (Engelmann et al., 2001), as verified in the VNTR sequence (Figure 1).

Discussion

The MUC1 VNTR sequence is a high GC content region as seen in Figure 1, causing great difficulty for PCR amplification, and requiring a special temperature balance between denaturing and annealing phases during each cycle. A long extension time is also important for long PCR fragments. True amplicons were all verified with DdeI restriction enzyme (PCR–RFLP) in order to eliminate the possibility of PCR artefacts (data not shown).

The average size for both smaller and larger alleles was not different between fertile and infertile women, contradicting Horne et al. (2001), who found a smaller size for infertile women. This may be due to the different ethnic composition of both populations, although both sample sizes were quite small.

Epithelial factors important in attachment may be regulated by paracrine interactions via the endometrial epithelium and the endometrial stroma, which is a progesterone-responsive tissue. This complex process involves a variety of molecules that are not unique in themselves, but play unique roles in the process of

Table 1. Number of repeats and allele sizes of the 60-bp VNTR MUC1 exon 2 region for infertile and fertile women.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Fertility classification</th>
<th>Estimated allele sizes (kb)</th>
<th>Alleles (estimated number of repeats)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Infertile</td>
<td>2.9/1.8</td>
<td>44–25</td>
</tr>
<tr>
<td>2</td>
<td>Fertile</td>
<td>2.9/1.8</td>
<td>44–25</td>
</tr>
<tr>
<td>3</td>
<td>Infertile</td>
<td>2.9/1.8</td>
<td>44–25</td>
</tr>
<tr>
<td>4</td>
<td>Fertile</td>
<td>1.6/1.6</td>
<td>22–22</td>
</tr>
<tr>
<td>5</td>
<td>Infertile</td>
<td>2.5/1.66</td>
<td>37–22</td>
</tr>
<tr>
<td>6</td>
<td>Fertile</td>
<td>2.4/1.66</td>
<td>36–23</td>
</tr>
<tr>
<td>7</td>
<td>Infertile</td>
<td>2.3/1.60</td>
<td>34–22</td>
</tr>
<tr>
<td>8</td>
<td>Fertile</td>
<td>2.9/1.70</td>
<td>38–24</td>
</tr>
<tr>
<td>9</td>
<td>Infertile</td>
<td>2.38/1.64</td>
<td>35–23</td>
</tr>
<tr>
<td>10</td>
<td>Fertile</td>
<td>1.7/1.70</td>
<td>24–24</td>
</tr>
<tr>
<td>11</td>
<td>Infertile</td>
<td>2.9/1.77</td>
<td>41–25</td>
</tr>
<tr>
<td>12</td>
<td>Fertile</td>
<td>2.5/1.70</td>
<td>37–24</td>
</tr>
<tr>
<td>13</td>
<td>Infertile</td>
<td>1.6/1.60</td>
<td>22–22</td>
</tr>
<tr>
<td>14</td>
<td>Infertile</td>
<td>2.5/1.69</td>
<td>36–24</td>
</tr>
<tr>
<td>15</td>
<td>Fertile</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>16</td>
<td>Fertile</td>
<td>2.5/1.70</td>
<td>37–24</td>
</tr>
<tr>
<td>17</td>
<td>Infertile</td>
<td>2.44/1.69</td>
<td>36–24</td>
</tr>
<tr>
<td>18</td>
<td>Infertile</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>19</td>
<td>Fertile</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>20</td>
<td>Infertile</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

Asterisk (*) indicates that fragments were not amplified under the long PCR conditions, although DNA was successfully amplified in a positive control reaction ($\beta_2$-microglobulin).
implantation (Giudice, 1999; Pellicer et al., 2002). Since implantation is hormonally controlled and is affected by many genes that have various polymorphisms, it is possible that there may be a genotypic disequilibrium among other genes, counterbalancing their unfavourable effects at the time of their expression, and depending on the endometrium cell microenvironment. However, one polymorphism must never be analysed alone without considering other key elements in the implantation process, such as integrins, osteopontin, calcitonin, progesterone receptors, tissue inhibitors of metalloproteinases (TIMPs), epidermal growth factors, cytokines, IGFBP1, and many others.

Modulation of endometrial receptivity was investigated during phases of the menstrual cycle and following treatment with progesterone antagonists, by studying the in-vivo expression of several molecular markers, such as progesterone and oestrogen receptors, cytokines, and cell adhesion molecules, but no direct correlation was observed among these markers, suggesting that there exists redundancy or multiple pathways that regulate implantation events (Puri et al., 2000).

It has been demonstrated in rabbits that MUC1 expression increases during the receptive phase but is locally reduced at sites of conceptus attachment (Hoffman et al., 1998). At the adhesion phase, the embryo induces a paracrine cleavage of the endometrial epithelial cell MUC1 at the implantation site. Human blastocysts also express MUC1, localized at the trophectoderm (Meseguer et al., 2001, Pellicer et al., 2002). MUC1 is up-regulated by progesterone and down-regulated in vitro by human blastocyst (Meseguer et al., 2001). The MUC1 proteolytic cleavage signalling during implantation may be associated with other genes that could be related to hormone receptors as well as to the intra- and extracellular calcium regulated balance. Because progesterone-induced expression of calcitonin in the secretory endometrium temporally coincides with the putative window of implantation in humans (Kumar et al., 1998), it has been postulated that calcitonin, a peptide hormone involved in calcium homeostasis, may play a role in preparing the apical cell pole for contact with the trophoblast (Giudice, 1999); however, this mechanism has not yet been demonstrated.

Parry et al. (2001) have demonstrated in in-vivo experiments that the tandem repeat does not influence the cleavage site. However, two naturally occurring isoforms of MUC1 that lack the tandem repeat array but comprise the N-terminal as well as the transmembrane and cytoplasmic MUC1 domains, MUC1/Y and MUC1/X have been shown not to be cleaved (Zrihan-Licht et al., 1994). Since MUC1/Y contains the signal sequence FRPG/SVVV, the lack of proteolytic cleavage of this molecule may be due to conformational changes in the structure of the isoform (Zrihan-Licht et al., 1994). The MUC1/Y and MUC1/X isoforms are differentially expressed in malignant epithelial cells, raising the possibility that they perform functions distinct from the full-length MUC1 and may not require cleavage (Baruch et al., 1997). These variations suggest that other MUC1 mutations may influence protein conformation affecting embryo implantation.

Post-translational modifications may also be affected by gene mutations, which could generate various degrees of glycosylation of the apomucin primary structure (core protein) as indicated by Gendler et al. (1990). Then, different glycosylation levels could lead to a lack of recognition of the cleavage site during the embryo paracrine signalling.

Although the present results lead to a lack of relationship of the 60-bp VNTR polymorphism of the MUC1 gene to female infertility or to embryo implantation failure, it is impossible to rule out its importance in the implantation regulatory cascade. Other genes are also involved in this complex process of implantation, probably masking the true significance of this variation. However, it is also possible that mutations within the MUC1 VNTR region may generate new isoforms with specific conformational changes that could prevent proteolytic cleavage or affect post-translational modifications of the glycoprotein.

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