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Sialyl-Lewis x and Sialyl-Lewis a are associated with MUC1 in human endometrium

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Endometrial epithelial cells express MUC1 with increased abundance in the secretory phase of the menstrual cycle, when embryo implantation occurs. MUC1 is associated with the apical surface of epithelial cells and is also secreted, being detectable in uterine fluid at elevated levels in the implantation phase. However, its physiological role is uncertain; it may either inhibit intercellular adhesion by steric hindrance or carry carbohydrate recognition structures capable of mediating cell-cell interaction. Here we show that endometrial epithelium expresses both Sialyl-Lewis x (SLe^x) and Sialyl-Lewis a (SLe^a), with a distribution and pattern of menstrual cycle regulation similar to that of MUC1. Using Western blotting and double determinant ELISA of uterine flushings, we demonstrate that SLe^x is associated with MUC1 core protein. The endometrial carcinoma cell lines HEC1A and HEC1B are shown to express MUC1 in a mosaic pattern, while three other cell lines express much lower amounts. HEC1A expresses both SLe^x and SLe^a while HEC1B expresses SLe^a only. Immunoprecipitation has been used to demonstrate that SLe^a is associated with MUC1 in HEC1B cells, and both SLe^x and SLe^a are associated with MUC1 in HEC1A cells.

Keywords: MUC1, Sialyl-Lewis x, Sialyl-Lewis a, endometrium, epithelium, carcinoma cells, secretion, implantation

Introduction

The molecular mechanisms that mediate the attachment of an embryo to the luminal epithelium of the endometrium, the initial stage of implantation, have yet to be elucidated. It is believed that the endometrium becomes receptive to interaction with an implanting blastocyst through steroidally regulated expression of cell surface componants capable of modulating the adhesive properties of the epithelium (reviewed in [1, 2]).

Ovulation at approximately mid cycle marks the onset of the secretory phase. The endometrial epithelial cell population undergoes progesterone-induced differentiation [3] and acquires a secretory phenotype in preparation for blastocyst implantation about 7–11 days later. An increase occurs in apically located Golgi apparatus and post-Golgi vesicular compartments [4]. This coincides with an increase in the level of glycosylation and gives rise to changes in the glycoprotein composition of the cell surface and secretions [5]. Endometrial glycans that demonstrate menstrual cycle-dependent patterns of expression include the sialokeratan structure recognized by McAb D9B1 [6, 7], keratan sulphate [8] and a blood group A-related epitope [9]. All three products appear maximally during the peri-implantation period, at the apical cell surface and also in lumenal secretions; furthermore all are associated with high molecular weight mucin-type molecules. It appears likely that the products of endometrial secretory differentiation provide a milieu favourable for implantation [10] and may be directly involved in this process [11].

The role of cell surface glycoconjugates in a variety of cellular recognition events is well documented. Most notably, the selectin family of receptor proteins mediate the adhesion of leucocytes to endothelial cells and platelets. The ligands are sialylated, sulphated and fucosylated oligosaccharides related to the SLe^x and SLe^a carbohydrate antigens. These are frequently associated with protein cores possessing mucin-like characteristics; domain regions rich in serine and threonine are potential sites of glycan chain attachment via O-glycosidic linkage [12]. Thus the possibility arises that

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mucin-linked glycans may play a role in blastocyst attachment.

We have reported the presence of the cell surface-associated mucin MUC1 in endometrial epithelium during the menstrual cycle [2, 13, 14]. MUC1 is a high molecular weight (300–400 kDa) molecule with a large extracellular domain containing variable number tandem repeat (VNTR) units of a conserved 20 amino acid sequence [15, 16]. Each repeat contains five potential sites of O-glycosylation and approximately 40% by weight is carbohydrate [7, 13]. Expression is regulated [17] with maximal levels in the mid secretory phase, around the peri-implantation period. Although the best-characterized *MUC1* gene product is a transmembrane molecule, expressed at the apical surface of endometrial epithelial cells [13], a large pool also exists as a component of glandular secretions [14].

Elevated MUC1 expression coincides with endometrial receptivity. This is a paradoxical observation as MUC1 has been shown to act as an anti-adhesion molecule, capable of inhibiting both cell-cell and cell matrix interactions *in vitro* [17, 18]. This property of MUC1 is probably conferred by its highly elongated and extended structure, which when expressed at high levels results in the masking of underlying adhesion receptors that do not extend as far beyond the cell surface. The predicted conformation of the molecule however also suggests that it could interact with ligands at apposing surfaces via carbohydrate recognition structures present on its many O-linked glycan chains.

Here we have examined the expression of the carbohydrate adhesion ligands SLe^x and SLe^a in endometrial epithelium throughout the menstrual cycle. We also report that these glycans are associated with endometrial MUC1. In addition we show distinct patterns of MUC1 and glycan expression by five endometrial carcinoma cell lines.

Materials and methods

Antibodies

Mouse monoclonal antibodies (McAb) BC2 (IgG) and BC3 (IgM) [19] were purchased from Medical Innovations Ltd, Queensland, Australia. Both antibodies react with similar peptide epitopes contained within the tandem repeat sequence of the MUC1 core protein. Monoclonal antibody 232A1 (IgG) reacts with a unique epitope in the extracellular domain of MUC1 [18]. This antibody was from Dr J. Hilkens, The Netherlands Cancer Institute, Amsterdam. Rabbit polyclonal antiserum CT1, raised against the cytoplasmic tail of MUC1 [20] was from Dr L. Pemberton, I.C.R.F. Lincolns Inn Fields, London.

Anti-SLe^x McAb AM3 (IgM) [21] was from Dr C. Hanski, Berlin, Germany. McAb 121SLE (IgM) against SLe^a [22] was from Dr J. Bara, Villejuif, France. McAb C50 (IgM) recognises SLe^a [23] but also the related unfucosylated terminal glycan [24]. It was purchased from CanAg Diagnostics, Gothenburg, Sweden.

Tissue Specimens

Endometrial tissue from curettage or hysterectomy was formalin-fixed, paraffin wax-embedded archival material provided by the Department of Histopathology, St Mary's Hospital, Manchester. Dating was based on the time of the last menstrual period and also by independant histological assessment according to the criteria of Noyes *et al.* [25]. In all cases used, endometrial dating and histology were consistant and tissue was reported free of neoplasia or infection.

Uterine flushings

Flushing of the uterine cavity with physiological saline provides a valuable method of recovering uterine secretions [26]. Uterine fluid contains the products of endometrial glandular secretions in addition to some plasma trans-exudate; it can therefore be used as a source of endometrial secretory glycans. All flushings used in this study were from a normal, fertile patient group previously described [14].

Immunohistochemistry

Sections (5 μm) were produced from 25 endometrial tissue specimens obtained in different phases of the menstrual cycle (seven from the proliferative phase and six each from early, mid and late secretory phases) and used for the immunohistochemical assessment of sialyl $Lewis^x$ expression in human endometrium.

Endogenous peroxidase activity was blocked by treatment with 1:100 (by vol) hydrogen peroxide in methanol and non-specific antibody binding blocked by 10 min incubation in Protein Block (Dako, High Wycombe, UK). Incubation in AM3 (neat hybridoma supernatent) was for 1 h at room temperature and followed by a similar incubation in biotinylated anti-mouse antibody (Dako) diluted 1:200 in PBS (pH7.6). An avidin-biotin amplification step was used as directed in the manufacturers instructions (Vectastain ABC Kit, Vector Laboratories, Peterborough, UK) and finally a Nickel-enhanced DAB substrate development kit (Vector) was used for the identification of immunoreactivity. Haematoxylin nuclear counterstaining was also performed on all sections.

Two sets of five sections (representing each phase of the cycle) were also selected for immunostaining with McAbs C50 and 121SLE. These antibodies were used at 1:250 dilution of ascitic fluid in PBS and neat culture supernatent respectively, to provide a comparison between SLe^x and SLe^a expression.

Western blotting

Uterine flushings were concentrated two-fold by dialysis and lyophilization. Reconstituted samples were denatured in reducing SDS sample buffer and separated by electrophoresis on 5% polyacrylamide gels. Pre-stained molecular weight markers (Biorad, Hemel Hempstead, UK) were included. Proteins were transfered to PVDF Immobilon membrane (Millipore Ltd, Watford, Herts, UK) by semi-dry electro-blotting. Transfer was performed for 2 h at 70 mA constant current. Membranes were blocked in 5% milk solids, 0.05% Tween-20 (Sigma, Poole, UK) in PBS for approximately 12 h and probed with McAbs BC3 (against MUC1 core protein) at 1:1000 dilution in PBS and AM3 (against SLe^x) at 1:2 dilution. All washes were with 0.05% PBS/Tween-20 (pH 7.6). A biotinylated anti-mouse secondary antibody (Dako) was used at a dilution of 1:200 and was detected by an avidin-biotin amplification kit (Vector). A coloured reaction product was generated using a Nickel-enhanced DAB substrate (Vector).

N-Glycanase digestion

Selected secretory phase uterine flushings (rich in MUC1 and associated SLe^x) were treated with N-glycanase prior to immunoblotting. Two identical aliquots, each containing approximately 25 μg of total protein, were lyophilised and reconstituted in 25 μl of 1 × incubation buffer (4 mM sodium phosphate pH 7.5, 10 mM EDTA, 0.02% sodium azide) (Oxford Glycosystems, Oxford, UK) containing 0.5% SDS and 5% mercaptoethanol. Samples were denatured by heating to 100 °C for 2 min, then cooled and 5 μl of 10% octyl glucoside (Sigma, Poole, UK) added. PBS (3.5 μl) was added to the control sample (mock-treated) and N-glycanase/peptide-N-glycosidase F (Oxford Glycosystems); 3.5 μl, ~0.7 U) to the test. Incubation at 25 °C was performed for 18 h, after which samples were concentrated by lyophilization and reconstituted in reducing SDS sample buffer. Electrophoresis and immunoblotting were performed as described above. N-glycanase-treated and control lanes were probed with BC3 and AM3.

Double determinant ELISA

This technique has been previously described [14, 27]. Briefly, anti-MUC1 core protein McAb BC2 (IgG) was used (purified IgG, 4 μg ml^{-1}) as a solid phase capture reagent. MUC1 retained from the specimen was then overlaid with a variety of detector antibodies of the IgM class: BC3 (ascitic fluid, 1:1000) to detect MUC1 core protein and AM3 (culture supernatant, 1:4) for the detection of SLe^x. In addition, C50 (ascitic fluid, 1:250) and 121SLE (ascitic fluid, 1:800) have been used to detect SLe^a carbohydrate antigens associated with MUC1. Finally, a peroxidase-conjugated anti-mouse IgM antibody (Nordic Immunological Laboratories, Maidenhead, UK) was applied and ABTS (Boehringer Mannheim, Lewes, Sussex, UK) used as chromogenic substrate. Absorbance was measured at 405 nm.

Removal of terminal sialic acid residues from MUC1 captured with BC2 was performed by incubating wells in a 1:100 dilution of Vibrio cholera neuraminidase (Boehringer) in 0.1 M sodium acetate, 1 mM calcium chloride for 1 h at 37 °C prior to incubations with detector antibodies.

Cell lines

Endometrial carcinoma cell lines HEC1A (American type culture collection, HTB-112) , HEC1B (HTB-113) and RL95-2 (CRL-1671) were from Dr Andrew Sharkey, University of Cambridge. HEC1As were maintained in McCoy's 5a medium (GibcoBRL, Paisley, UK) containing 10% foetal bovine serum (FBS) (Advanced Protein Products, Brierley Hill, West Midlands, UK). HEC1Bs were maintained in Eagles MEM supplemented with Earle's balanced salt solution, 10% FBS and 1 mM sodium pyruvate (GibcoBRL). RL95-2 cells were maintained in a 1:1 mixture of DMEM and Ham's F12 containing 10 mM HEPES (GibcoBRL), 5 μg ml^{-1} insulin, 2 g l^{-1} $NaHCO_3$ and 10% FBS. AN3-CA (HTB-111) were from Dr Michael Thie and Dr Elke Winterhager, Institut fur Anatomie, Universität Klinikum, Essen and were maintained in Eagle's MEM with Earles salts and non-essential amino acids (Sigma) with 10% FBS supplementation. The Ishikawa cell line was from Dr John White, Hammersmith Hospital, London; these cells were maintained in DMEM containing 20 mM HEPES (GibcoBRL) and 10% FBS. In addition all media was supplemented with glutamine (2 mM) (ICN, Thame, Oxon, UK) and antibiotics: streptomycin 100 μg ml^{-1} (Evans Medical Ltd, Horsham, UK) and gentamycin 5 μg ml^{-1} (David Bull Laboratories, Warwick, UK). All cells were grown in monolayer culture in an atmosphere of 95% air: 5% carbon dioxide. For immunofluorescence, confluent monolayers were grown on 19 mm diameter glass coverslips (Chance Propper Ltd., Smethwick, UK) in plastic 12 well plates (Corning, Corning, NY, USA).

Immunofluorescence

Confluent monolayers were fixed sequentially in 3.7% formaldehyde in PBS (pH 7.6) and methanol, for 20 min each at room temperature. Indirect immunofluorescence was performed using McAb BC3 (1:500 in PBS) against MUC1 core protein and McAbs AM3 (neat hybridoma supernatant) and C50 (1:250 in PBS) against SLe^x and SLe^a carbohydrate antigens respectively. Primary antibody was detected with rabbit anti-mouse conjugated to fluorescein isothiocyanate (FITC) (Dako, High Wycombe, UK) at a dilution of 1:50 in PBS in the dark. Coverslips

were mounted in aqueous Immu-mount (Shandon, Warrington, UK) and viewed on a fluorescence microscope.

Immunoprecipitation

Confluent HEC1B cells, grown in 75 cm^2 flasks (Costar UK Ltd, High Wycombe, Bucks, UK) were incubated in Eagles MEM without glucose (GibcoBRL) and FCS for 2 h prior to labelling. Cells were labelled in MEM without glucose, containing 100 μCi per flask D-[1,6-^{3}H]-glucosamine hydrochloride (NEN Research Products, Stevenage, UK) for 16 h. Medium was removed and the cell monolayer washed twice in cold PBS (pH 7.6). Cells were scraped into PBS with a sterile policeman (Costar) and treated with lysis buffer (5 mg ml^{-1} BSA, 1% sodium deoxycholate, 25 $\mu g\,ml^{-1}$ leupeptin, 1 mM AEBSF (ICN; all others from Sigma) 2 mM NEM in PBS, pH 7.6). This extract was spun at 12 000 × **g** in a microfuge for 5 min and the supernatant pre-cleared by the addition of rabbit anti-mouse immunoglobulins (Dako) and Protein A beads (Sigma) for 2 h at 4 °C. The beads were then pelleted by centrifugation and discarded. Protein-A beads to be used in the immunoprecipitation were pre-blocked by overnight incubation at 4 °C with cold cell extract and pre-loaded with rabbit anti-mouse immunoglobulins (Dako) to a 1:50 dilution during a second overnight incubation at 4 °C. Preloading was performed because BC3 and C50 are IgMs and are not retained by Protein A. Immunoprecipitation from 200 μl aliquots of labelled extract was initiated by overnight incubation at 4 °C with 5 μl BC3, 40 μl AM3, 40 μl 232A1, 5 μl C50 and 5 μl CT1. Controls in which no precipitating antibody was added were also included. Sixty μl of pre-blocked, pre-loaded Protein A beads, (diluted 1:1 with PBS) was added to each reaction and incubated at room temperature for 3 h. Protein A beads were pelleted by centrifugation and after extensive washing in 0.3% Triton X-100 (Sigma)/PBS, were resuspended in reducing sample buffer (TrisHCl pH 6.7 containing 4% SDS 20% glycerol, 10% 2-mercaptoethanol and 0.4% Bromophenol blue) in a 1:1 ratio, boiled for 5 min and electrophoresed on a 2–9% polyacrylamide gradient gel. Gels were fixed in ethanol:formaldehyde:-distilled water (51:29:120) for 30 min and soaked in Amplify solution (Amersham International, Little Chalfont, Bucks., UK) to enhance the tritium-generated autoradiographic signal. Gels were dried under vacuum and exposed to pre-flashed Hyperfilm MP (Amersham) for 1–2 weeks at −80 °C.

Results

SLe^x and SLe^a are expressed in endometrial epithelium

Immunohistochemistry using McAb AM3 was performed to examine the expression of SLe^x carbohydrate antigen in endometrium throughout the menstrual cycle. In proliferative phase tissue immunoreactivity was predominantly located within the apical cytoplasm of both glandular and luminal epithelial cells, in a peri-nuclear distribution (Fig. 1a). In addition, immunoreactive basal deposits were also present in a small number of cells. Staining of the apical surface of both glandular and luminal epithelial cells was observed, with occasional immunoreactivity in sparse gland secretions. Some inter-gland and intra-gland heterogeneity in antigen expression was evident, with negative gland cells interspersed amongst positives.

In early secretory phase endometrium a prominent concentration of supranuclear staining was observed within the cytoplasm of epithelial cells. Overall, antigen expression appeared less heterogeneous than in proliferative phase tissue, with a slight increase in the volume of immunopositive secretions (Fig. 1b). In the mid secretory phase, cytoplasmic immunoreactivity became more diffuse with an overall increase in the level of staining, which was in general less heterogeneous. Positive secretions were also prominent in the gland lumens (Fig. 1c). Variation in the level of immunostaining between tissue specimens was however also apparent. Staining of late secretory phase endometrial tissue was also characterised by heterogeneity between specimens. In addition, considerable heterogeneity within glands was observed with many glands containing strongly stained cells amongst weaker, more diffusely stained neighbours. Immunoreactive glandular secretions remained, but some glands also contained secretory material that was not immunoreactive (Fig. 1d).

Immunohistochemistry was also performed using McAbs C50 and 121SLE to compare the distribution of SLe^a with that of SLe^x in sections of endometrium selected from different phases of the menstrual cycle. Small areas of epithelial immunoreactivity were detected in proliferative phase tissue with both C50 and 121SLE (not shown). In general levels were lower than with AM3. In the early and mid secretory phase, more SLe^a immunoreactivity became evident in glandular and luminal epithelium, although staining was never as extensive as with McAb AM3. Figure 2 shows adjacent sections of late secretory phase endometrium stained with anti-MUC1 core protein McAb BC3 (Fig. 2a), anti-SLe^x McAb AM3 (Fig. 2b), anti-SLe^a McAb C50 (Fig. 2c) or anti-SLe^a McAb 121SLE (Fig. 2d). In each case immunoreactivity was confined to the epithelial cells, both luminal (not shown) and glandular and their secretions. With each antibody, staining was observed in the epithelial cytoplasm as well as at apical cell surfaces. Considerable heterogeneity was present between adjacent cells and between different gland profiles. Reduced levels of immunostaining were again observed with C50 relative to AM3, suggesting lower levels of SLe^a expression relative to SLe^x. Significantly lower levels of immunostaining were observed with 121SLE than with C50

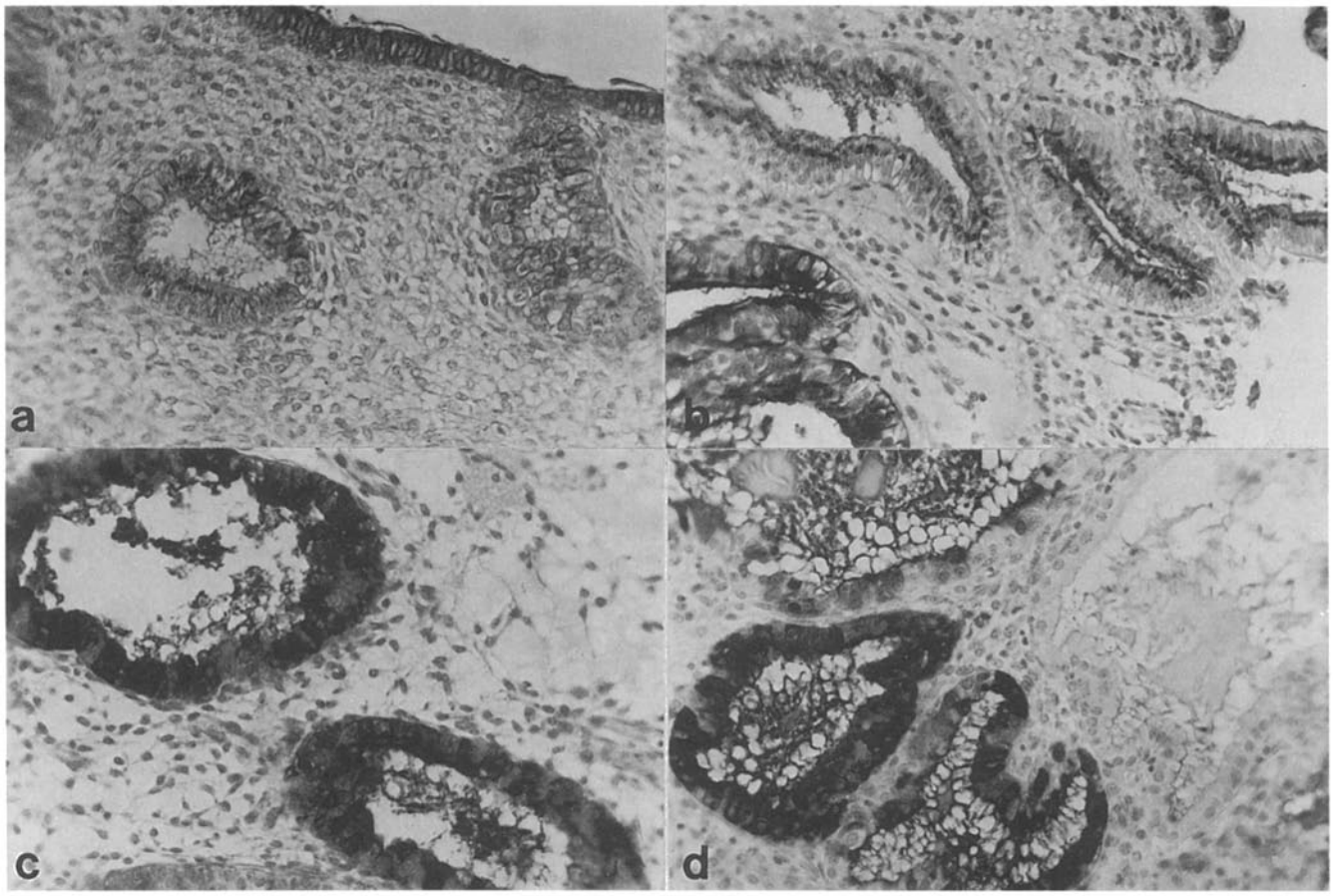

Figure 1. Immunolocalization of SLe^x in normal endometrium during the menstrual cycle using McAb AM3. (a) Proliferative phase showing weak immunoreactivity in the luminal epithelium (top) and two glands. (b) Early secretory phase glands showing supranuclear accumulations of reactivity as well as apical cell surface staining. (c) Two mid secretory phase glands with intense cytoplasmic and secretory reactivity. (d) Late secretory phase glands showing strong staining, weaker staining and (top left) one immunonegative gland containing abundant secretory material (×200). No staining is seen in the stroma.

(Fig. 2c,d), suggesting subtle differences in the structure of epitopes recognised by these two anti-SLe^a antibodies ([24]).

Endometrial carcinoma cell lines exhibit distinct patterns of glycan and MUC1 core protein expression

Indirect immunofluorescence was used to investigate the expression of MUC1 core protein and the SLe^x and SLe^a carbohydrate antigens by five endometrial carcinoma cell lines grown in monolayer culture: AN3-CA, RL95-2, HEC1A, HEC1B and Ishikawa.

HEC1A cells displayed a heterogeneous pattern of primarily punctate fluorescent staining with the anti-MUC1 McAb BC3 (Fig. 3a). Labelled cells exhibited a range of intensities suggesting heterogeneity in the level of MUC1 expression throughout the population. A small number of cells appeared completely negative. A different pattern of fluorescence was observed with McAb AM3 to SLe^x (Fig. 3b). A significant proportion of cells were intensely labelled and often located together in large groups. These cells were more uniformly stained and did not display the range of fluorescence intensities observed with BC3. However distinct areas of weakly labelled cells were also evident within the monolayer, in addition to a small proportion of unlabelled cells.

Monoclonal antibody C50 detected a heterogeneous distribution of cells expressing the SLe^a antigen (Fig. 3c). A range of fluorescence intensities was observed, reflecting variation in the level of expression between cells in the monolayer. Unlike with McAb AM3, large homogeneous areas of intense fluorescence were not present. Again, a small proportion of negative cells was observed.

HEC1B cells exhibited similar patterns of fluorescent staining to HEC1As when screened for MUC1 core protein and SLe^a expression. However, this cell line did not express SLe^x (data not shown).

The Ishikawa cell line produced a heterogeneous pattern of fluorescence with BC3. The intensity of staining was lower than that observed with HEC1A and

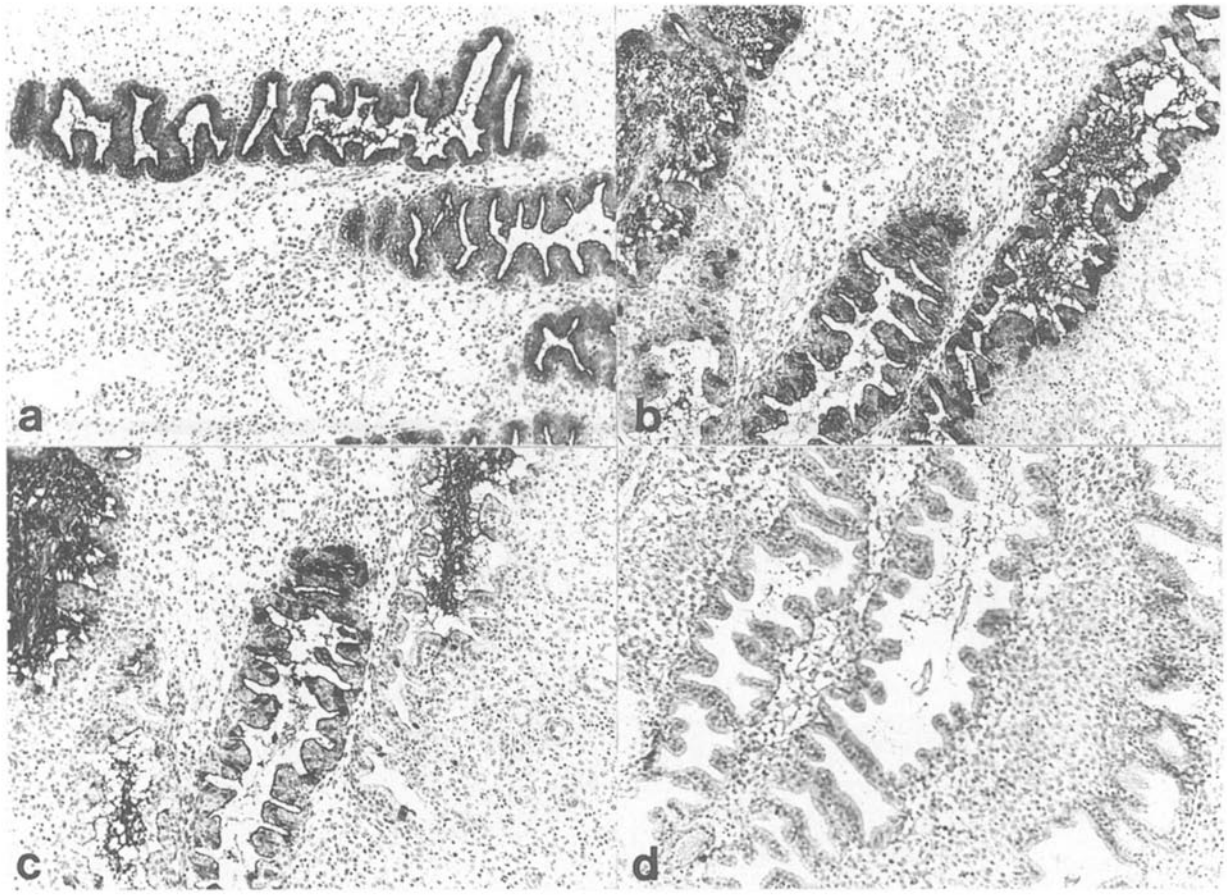

Figure 2. Immunoperoxidase localisation of MUC1, SLe^a and SLe^x in a specimen of late secretory phase endometrium. (a) MUC1 core protein (McAb BC3) at the apical surface of gland cells, in secretions and in the epithelial cytoplasm. (b) SLe^x (McAb AM3) similarly distributed. Note the inter-glandular and inter-cellular heterogeneity. (c) SLe^a (McAb C50) also shows a similar distribution. (d) McAb 121SLE also recognises SLe^a, but no binding is detectable with this antibody. Serial sections, ×80.

HEC1B cell lines; a higher proportion of negative cells were also evident. McAb AM3 identified a small number of cells within the monolayer that appeared to be expressing high levels of the SLe^x antigen. These cells were intensely fluorescent and clearly discernible. However, there was a degree of heterogeneity amongst the less intensely stained cells. This same distribution of fluorescence staining was also observed with McAb C50 suggesting similar levels of expression of SLe^x and SLe^a antigens in this cell line.

RL95-2 cells did not express MUC1. A very small number of these cells strongly expressed the SLe^x antigen, producing intense, punctate fluorescent staining. However, the majority of cells in the culture contained only very small foci of staining which appeared to be peri-nuclear in distribution (Fig. 3d). AN3-CA cells did not express MUC1 core protein nor the SLe^a or SLe^x antigens. These data are summarized in Table 1.

SLe^x and SLe^a are associated with MUC1 core protein

Western blotting of concentrated uterine flushings was carried out in order to establish the presence of MUC1 in the uterine lumen and to investigate the possibility that SLe^x and SLe^a are present as associated glycans. Probing with McAb BC3 revealed two major bands at approximately 400 kDa, presumably representing the two alleles of the MUC1 core protein (Fig. 4a,b). McAb AM3 gave an identical pattern of high molecular weight immunoreactivity. Comigration of SLe^x and MUC1 was observed in samples of secretory phase flushings from four different

Table 1. Summary of immunolocalization data obtained from 5 endometrial carcinoma cell lines.

Antibody cell line	*BC3 (MUC1 core protein)*	*AM3 (sialyl-Lewis^x)*	*C50 (sialyl-Lewis^a)*
AN3-CA	–	–	–
HEC1A	++	+++	++
HEC1B	++	–	++
Ishikawa	+	+	+
RL95-2	–	+	ND

–, negative; +, positive (small proportion of cells labelled); +++, positive (large proportion of cells labelled); ND, not determined.

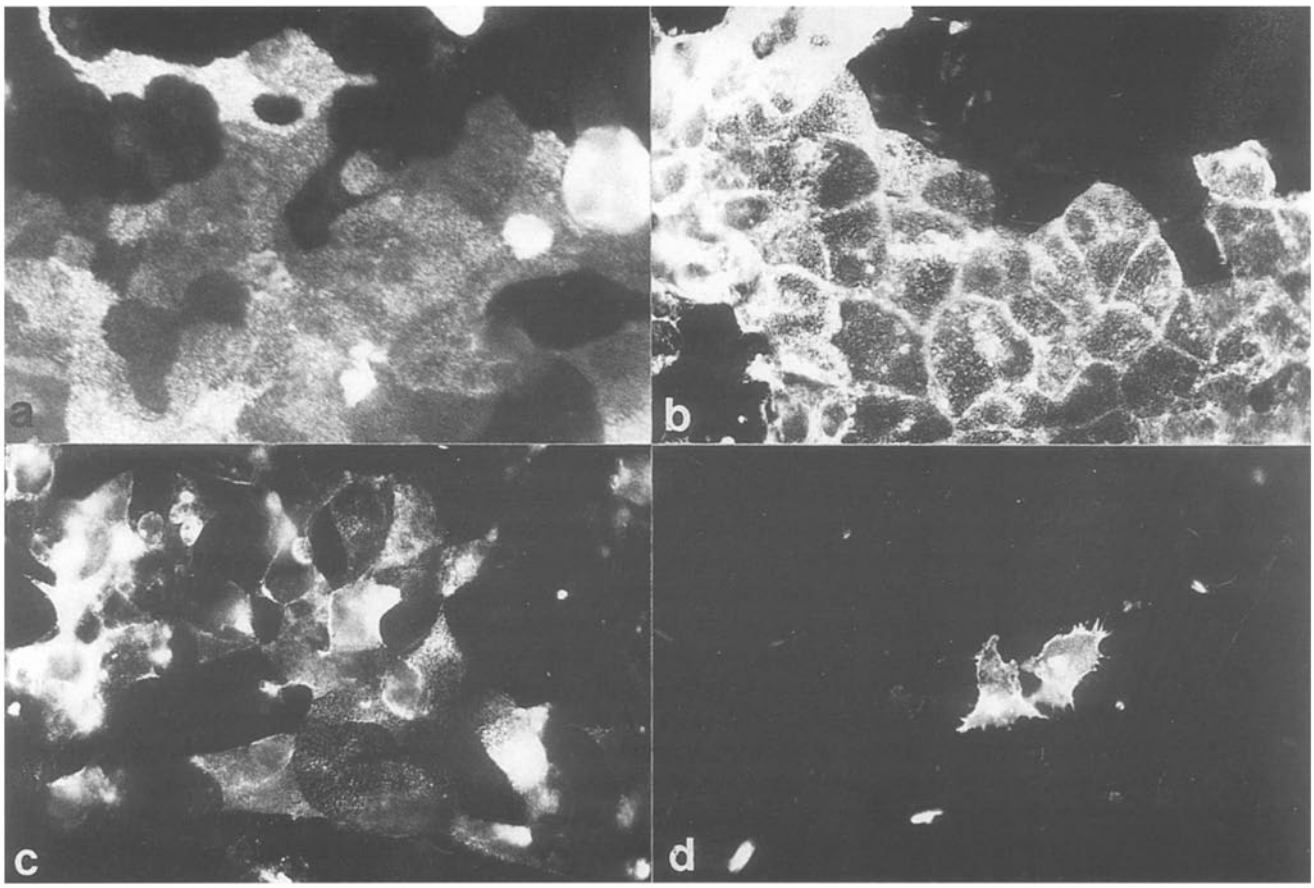

Figure 3. Immunofluorescence localisation of MUC1, SLe^a and SLe^x in cultured endometrial carcinoma cells in confluent monolayer culture. (a) MUC1 core protein (McAb BC3) in HEC1A cells, showing mosaicism; the staining intensity ranges from strong to negative. (b) SLe^x (McAb AM3) in HEC1A. Again there is a mosaic, but the immunopositive cells are clustered together. (c) SLe^a (McAb C50): strongly positive, weakly positive and negative cells are scattered apparently at random. (d) SLe^x (McAb AM3) in RL95 cells. This cell line does not express detectable MUC1 core protein. A few cells do express SLe^x strongly, others have focal intracellular reactivity. ×400.

individuals using Western blotting. This suggests that SLe^x is associated with MUC1 core protein. N-glycanase digestion of uterine flushings resulted in no detectable loss of AM3 signal on subsequent Western blot (Fig. 4c,d).

Uterine flushings were also analysed by double determinant ELISA in which McAb BC2 was attached to a solid phase and used to capture MUC1. Capture was confirmed using McAb BC3 to detect MUC1 core protein on the solid phase. Antibodies AM3 and C50 to SLe^x and SLe^a respectively were bound on the solid phase, thus confirming the association of both of these structures with MUC1 (Fig. 5). As expected from the immunohistochemical observations, a larger signal was observed for SLe^x than for SLe^a. The SLe^a observations were confirmed in specimens of secretory phase flushings from 14 different women, while the SLe^x observations were consistent in 15 individuals.

Post-capture enzymic desialylation of MUC1 resulted in the abolition of AM3 binding. This confirmed the specificity of the detection step, the sialic acid moiety being an essential componant of the epitope. In contrast, there was a net absorbance increase of 37% ($n = 3$; range = 31.5–43.5%) with BC3 as detector (data not shown). The removal of terminal sialic acid residues appeared to result in exposure of core protein epitopes that were previously masked.

Immunoprecipitation of MUC1 core protein and SLe^a was performed from detergent extracts of [3H]glucosamine-labelled HEC1B cells. McAbs BC3, 232A1 and CT1, all of which bind to different epitopes on MUC1 core protein, precipitated similarly migrating high molecular weight doublets from HEC1B (Fig. 6a,d,f). McAb C50 precipitated a comigrating doublet (Fig. 6c). McAb AM3 failed to precipitate anything, confirming the immunostaining data. Immunoprecipitations were also carried out from HEC1B conditioned culture medium (Fig. 7). McAbs BC3, C50 and 232A1 all precipitated comigrating high molecular weight species. However, no

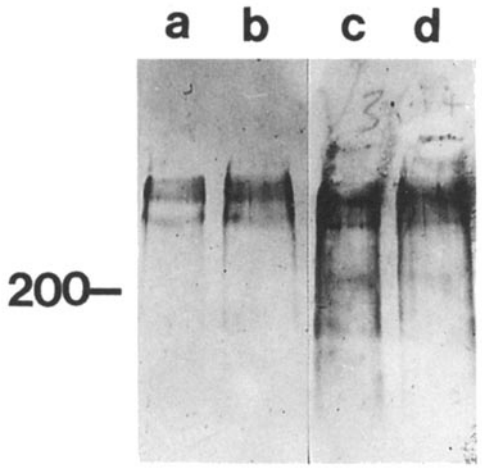

Figure 4. Western blots of uterine flushing probed with McAbs (a,b) BC3 or (c,d) AM3. (a,c) N-glycanase-treated; (b,d) mock-treated. Each lane was loaded with 25 μg protein from the same specimen. Note the prominent doublet at high M_r representing MUC1 and associated SLe^x. There is no change in antibody binding after N-glycanase treatment.

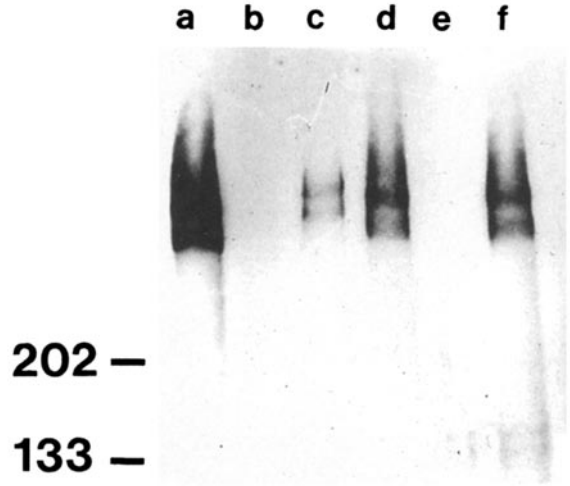

Figure 6. Immunoprecipitation from detergent-extracted [3H]glucosamine-labelled HEC1B cells. McAbs: (a) BC3 (MUC1 VNTR); (b) AM3 (SLe^x); (c) C50 (SLe^a); (d) 232A1 (MUC1 ectodomain); (e) control; (f) CT1 (MUC1 cytoplasmic tail).

bands were detected when CT1 was used, suggesting that the secreted form of MUC1 lacks the cytoplasmic domain.

Immunoprecipitation from detergent extracts of labelled HEC1A cells generated similar results to the HEC1B cells (Fig. 8). However, in this case, AM3 also precipitated a comigrating band, consistent with the immunostaining data.

Discussion

In previous work we have shown that MUC1 is expressed in endometrial epithelium in a hormonally-regulated pattern, with low levels of mRNA and core protein in

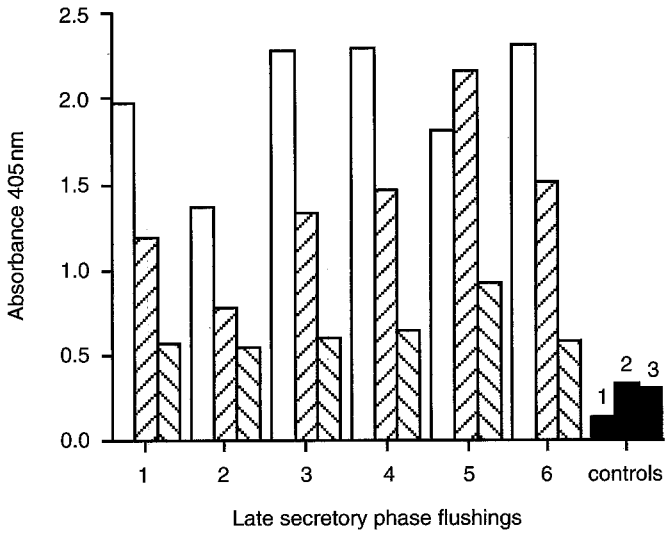

Figure 5. Capture-detect ELISA of six speciments of late secretory phase uterine flushings. McAb BC2 to MUC1 was used to bind mucin to the solid phase. Detection with McAb BC3 to MUC1 (open bars) confirmed that capture had occurred. McAbs AM3 (left hatch) and C50 (right hatch) were used to detect MUC1-associated SLe antigen. Controls (filled bars): 1, no capture antibody; 2, no detector antibody; 3, no antigen.

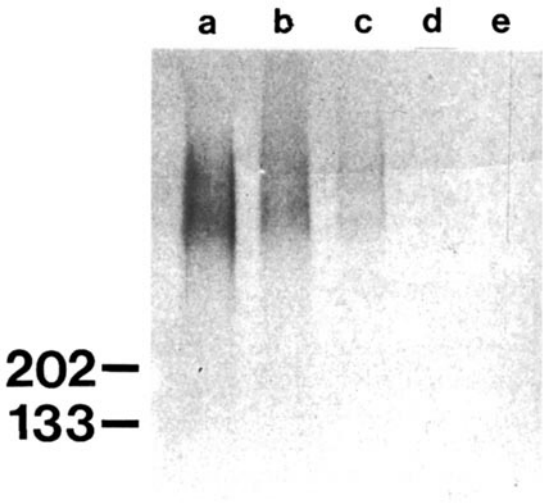

Figure 7. Immunoprecipitation from conditioned culture medium from [3H]glucosamine-labelled HEC1B cells. McAbs: (a) BC3 (MUC1 VNTR); (b) C50 (SLe^a); (c) 232A1 (MUC1 ectodomain); (d) control; (e) CT1 (MUC1 cytoplasmic tail).

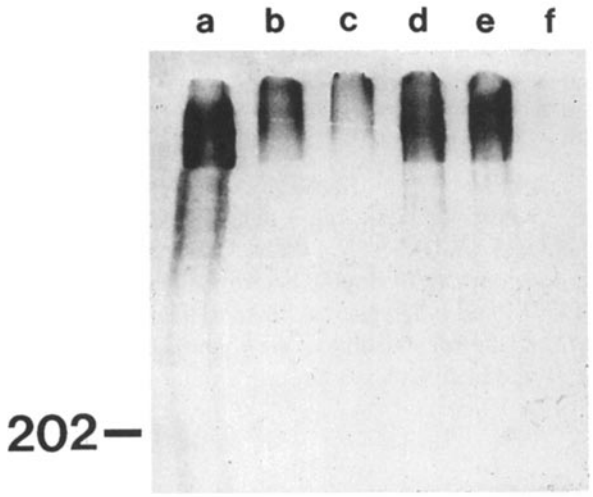

Figure 8. Immunoprecipitation from detergent-extracted [3H]glucosamine-labelled HEC1A cells. McAbs: (a) BC3 (MUC1 VNTR); (b) AM3 (SLe^x); (c) C50 (SLe^a); (d) 232A1 (MUC1 ectodomain); (e) CT1 (MUC1 cytoplasmic tail); (f) control.

the proliferative phase and an increase after ovulation. In the early secretory phase (days 15–18), mRNA abundance increases approximately six-fold and the core protein appears in increased amounts in the cytoplasm of glandular and luminal epithelial cells and at the apical epithelial cell surface. In the mid secretory phase (days 19–22), mRNA abundance remains high; at this stage the core protein is secreted, appearing in greatly increased amounts in gland lumens. In the late secretory phase, cell-associated MUC1 is still present but in diminished amounts; increased intercellular heterogeneity is observed in the epithelium, some cells being strongly immunopositive while others are negative. At this stage secretory material is still quite abundant [13]. Secretory MUC1 appears in the uterine luminal fluid where concentrations show a dramatic increase between days 21 and 24 [14]. This is the time when embryo implantation is expected to occur. Thus the appearance of MUC1 in uterine flushings is a possible molecular marker of the receptive phase for implantation.

Others have observed a secretory isoform of MUC1 in breast carcinoma cell culture supernatants [20, 28] and we have now confirmed that endometrial carcinoma cell lines also release soluble MUC1. We are currently investigating the mechanism whereby large amounts of MUC1 are released from endometrial cells in a hormonally-regulated fashion.

Previous studies have indicated that glycosylation in general and sialylation in particular are highly cycle-dependent in endometrial tissue, especially in the epithelium [3, 5, 6–9, 29–31]. In the present study we have shown that two sialoglycan epitopes are regulated. Both SLe^a and SLe^x are epithelial-specific products; both are upregulated strongly in the secretory phase. Furthermore, their distribution in secretory phase tissue was observed to be very similar to MUC1, suggesting the possibility that both may be present on the MUC1 core protein. Ravn *et al.* [29] observed the presence of Lewis^x in normal endometrium and showed an increase in antibody binding in the secretory phase after tissue sections were treated with neuraminidase, thus suggesting that masking by sialic acid was occurring. They also showed the presence of SLe^a in endometrium and its increase in the secretory phase [30]. Others have observed both antigens in association with MUC1 in cultured colon carcinoma cells [23, 32] and SLe^a has been observed in association with MUC1 from bile [33].

We have confirmed the association between MUC1 and sialyl-Lewis antigens in normal endometrium and in cultured endometrial carcinoma cell lines. Uterine flushings provided a convenient source of normal secretory MUC1 for biochemical analysis; comigration of immunoreactive bands in Western blots and double determinant ELISA measurements using anti-core protein and anti-glycan McAbs both indicate that SLe^x is displayed on MUC1. SLe^a appears to be less abundant based on ELISA and immunohistochemistry. However, immunoprecipitation indicated that MUC1 carries SLe^a in the HEC1B cell line. The absence of MUC1 core protein from two (ANC-3A, RL95) of the five endometrial cell lines examined, and the low level of its expression in a third (Ishikawa) suggests that caution should be exercised in the use of these lines for modelling embryo-epithelial interactions *in vitro*.

The epitopes recognized by antibodies BC2 and BC3 are situated in the tandem repeat region of the core protein [34]. The TR contains 20 amino acid residues of which five are Ser or Thr and at least two of the Thr residues are utilized [35]. MUC1 alleles contain between 20 and 100 repeat sequences [15]. Thus this region contains multiple potential O-glycosylation sites. The apparent M_r of endometrial MUC1 of approximately 400 kDa confirms that it is extensively glycosylated since even the largest alleles are likely to produce core polypeptides of only about 225 kDa. There is extensive evidence that glycosylation affects the binding of antibodies to the polypeptide [36]. Indeed, we have shown that desialylation of normal endometrial MUC1 leads to increased binding of McAbs to the core protein [13]. This suggests the likelihood that the sialyl-Lewis structures are O-linked. However, MUC1 also contains N-glycans, attached at one or more of five consensus tripeptides in the membrane-proximal part of the ectodomain [37]. N-glycanase treatment gave no significant alteration in the binding of AM3 to endometrial MUC1. Although this result supports SLe^x association via

O-linkage, further experiments will be required both to demonstrate conclusively the linkage type and to map specific glycans within the ectodomain.

It is not clear whether the functional role of MUC1 in endometrium is at the cell surface or as a secretory component of uterine fluid. It seems clear that MUC1 is present at the apical epithelial surface at the time of embryo attachment. However, variations are evident in the level of expression between different cells, giving rise to a mosaic pattern. This is observed in cultured carcinoma cells and primary cultured epithelial cells ([38] and unpublished data) as well as *in vivo*. The area of interaction between embryo and epithelium may initially be small [39, 40] and so attachment might occur either in MUC1-rich or MUC1-poor microdomains of the luminal epithelium.

There is experimental evidence to suggest that uterine glycans may play a direct role in the implantation process. Studies in the mouse have identified an H type I carbohydrate structure, lacto-*N*-fucopentaose I (LNF-I) that is expressed on uterine epithelium under hormonal control. H type I appears to be involved in the initial interaction between blastocyst and the uterine surface [11]. Lactosaminoglycans have also been demonstrated to be under steroidal regulation in both mouse [41–43] and rat [44]. Zhu *et al.* [45] have demonstrated the expression of Le^y oligosaccharide on both mouse blastocyst and uterine epithelial surfaces. Interaction of Le^y with H type I was proposed to promote close apposition of blastocyst and uterine epithelium during the initial stages of implantation. Data from *in vitro* heterologous cell-cell adhesion assays show that initial attachment of trophoblast derived human choriocarcinoma cells (JAR) to RL95-2 endometrial carcinoma monolayers is mediated by cell surface glycosaminoglycans with heparin-like properties [46]. The expression of the heparan sulphate proteoglycan perlecan on the trophectoderm surface correlates with attachment competence of mouse embryos *in vitro* [47].

The presence of SLe^x and SLe^a on cell surface MUC1 suggests the possibility of a selectin-mediated mechanism [11] and indeed we have observed that L-selectin is expressed at early preimplantation stages of human embryogenesis [48]. However, based on the observation that MUC1, when present at high levels at the cell surface, can sterically hinder access of adhesion molecules to their ligands [18, 49] there is also the possibility that MUC1 may act to inhibit embryo attachment except in spatially and temporally restricted regions of the epithelial mosaic where the level of inhibitor is low and interaction can occur with adhesion receptors of the integrin or other families [2]. More detailed knowledge of the distribution and glycosylation of MUC1 at the luminal epithelial surface in the peri-implantation phase, together with much needed analysis of human trophectodermal surface composition, should allow the refinement of current functional models.

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