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Human endometrial MUC1 carries keratan sulfate: characteristic glycoforms in the luminal epithelium at receptivity

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MUC1 is a high molecular mass, highly glycosylated epithelial apical glycoprotein that has been shown to exhibit both adhesive and anti-adhesive properties. Its expression in human glandular endometrial epithelium is transcriptionally regulated with the highest levels in the mid secretory phase, the "receptive" period during which implantation occurs. We demonstrate that endometrial MUC1 carries highly sulfated lactosaminoglycan chains recognized by monoclonal antibody (Mab) 5D4, and the sialokeratan sulfate epitope recognized by Mab D9B1. These glycans are hormonally regulated in endometrium, and show increased abundance in the secretory phase, but detailed evaluation of their distribution shows important differences. The 5D4 epitope is abundant at the luminal epithelial surface until the implantation phase, when it disappears, first from patches of cells, then altogether. D9B1 binding sites are retained in the luminal epithelium at receptivity. These data show that endometrial MUC1 carries sulfated lactosaminoglycans. They identify the luminal epithelial compartment as a site of unique MUC1 glycosylation and independent regulation. Glycosylation and the negative charge associated with sialo- and sulfoglycans may be important in the regulation of embryo attachment.

Key words: endometrium/glycosylation/implantation/mucin/MUC1

Introduction

Human implantation requires a sequence of events beginning with attachment of a blastocyst to the endometrial luminal epithelium and leading to its penetration of the maternal stroma and access to the vasculature. The molecular mechanisms are largely unknown. Direct evidence from rodent systems indicates the existence of a receptive phase produced in endometrium by the action of maternal steroid hormones (reviewed in Aplin, 1996). Data produced by fertilization *in vitro* followed by timed embryo transfer suggest a similar phenomenon in human (Bergh and Navot, 1992).

Glycans and their cognate binding proteins have been suggested to play a role in mediating the initial interaction between blastocyst and endometrial epithelium. In mice, H type 1 is maternally expressed and has a receptor on the blastocyst (Lindenberg *et al.*, 1988; Kimber, 1994); heparan sulfate is present at the cell surface, and a high affinity binding protein has been demonstrated in endometrium (Carson *et al.*, 1993, 1995); anti-Lewis y antibodies have been shown to inhibit implantation (Zhu *et al.*, 1995); and sialyl Lewis x and sialyl Lewis a are abundant in human endometrial epithelium in the secretory phase (Hey and Aplin, 1996).

One way in which a receptive phase could be controlled is to modulate an adhesion system at the cell surface. This could involve the regulated appearance of an adhesion molecule or possibly the unmasking of a constitutively expressed receptor. The apical glandular epithelial surface of the human endometrium has been shown to contain abundant quantities of the cell surface mucin MUC1 (Hey *et al.*, 1994). In the midsecretory, implantation phase, MUC1 is also secreted into gland lumens and appears in the uterine luminal fluid (Hey *et al.*, 1995). Overexpression of MUC1 at the cell surface gives rise to steric inhibition of intercellular adhesion in cultured cells (Hilkens *et al.*, 1992; Wesseling *et al.*, 1995, 1996), and it has been characterized as an anti-adhesion molecule the upregulation of which in tumors might encourage metastatic spread. In mouse, MUC1 is present at the apical endometrial epithelial surface in estrous phase and the first 2 days of pregnancy, but its abundance declines steeply in the implantation phase (Braga and Gendler, 1993). This has led to suggestions that its loss may expose adhesion molecules that mediate attachment (Hey *et al.*, 1994; Surveyor *et al.*, 1995; Aplin, 1996). In human endometrium, however, neither MUC1 mRNA nor core protein declines in abundance in mid secretory phase; indeed, the abundance of the transcript increases 6-fold compared to proliferative phase levels (Hey *et al.*, 1994). However, it has been demonstrated that MUC1 can interact via its variable number tandem repeat (VNTR) region with ICAM1 and that this interaction is capable of mediating cell adhesion (Regimbald *et al.,* 1996).

MUC1 has an unusually long ectodomain, most of which comprises a VNTR rich in serine, threonine, glycine, and proline. It is polymorphic, with allelic differences arising by variation in the number of repeats; individuals codominantly express both alleles. It is predicted to assume a conformation that extends much farther from the cell surface than adhesion receptors of the families mentioned above. In endometrium about 40% of its molecular mass is predicted to be carbohydrate, and the possibility arises that MUC1 acts as a scaffold upon which glycan recognition structures could be mounted. Such structures might exhibit a pattern of temporally or spatially regulated expression independent of core protein regulation, and this might allow a role in implantation. In recent work we have demonstrated that endometrial MUC1 bears sialyl Lewis x and sialyl Lewis a, both of which have been associated with selectin-mediated adhesion (Hey and Aplin, 1996). We have shown that sialylation of endometrial MUC1 increases in the secretory phase (Hey *et al.*, 1994). By raising monoclonal antibodies (Mabs) to hormonally modulated cell surface components, we have also defined a family of sulfated sialylated lactosaminoglycans that is expressed

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in glandular epithelium at peak concentrations in the midsecretory phase and is associated with a high molecular weight glycoprotein (Smith *et al.*, 1989; Hoadley *et al.*, 1990; Aplin, 1991; Graham *et al.*, 1994). Others have reported that the sialyl Tn antigen (NeuNAcα2–6GalNAc), commonly associated with mucins, is modulated, with no detectable expression in the proliferative phase and maximal expression in midsecretory phase glands (Thor *et al.*, 1987). Similarly, a mucin-associated blood group A-related structure is upregulated in secretory phase glands (Kliman *et al.*, 1995; Jones *et al.*, 1997).

We now show that several of these hormonally modulated glycans are associated with endometrial MUC1. We further demonstrate that the luminal epithelial cell compartment, where initial interaction with the blastocyst occurs at implantation, exhibits a unique pattern of MUC1 glycosylation, and therefore represents a distinct differentiation state within the epithelial population. Finally, we suggest that microdomains may exist in the luminal epithelium where differential adhesive properties might be found.

Results

The glycans detected by Mabs D9B1 and 5D4 are associated with MUC1

Mab D9B1 has previously been shown to recognize a sialoglycan epitope associated with a high molecular mass glycoprotein that bands as a doublet in SDS–PAGE (Hoadley *et al.*, 1990). Figure 1A shows a Western blot of endometrial tissue extracts probed with D9B1 or the anti-MUC1 core protein Mab HMFG1. Both antibodies recognize the same comigrating doublet.

Figure 1B shows a similar experiment in which the anti-keratan sulfate Mab 5D4 is compared with the anti-MUC1 core protein Mab BC3, again showing comigrating doublets. The results suggest that keratan sulfate and the sialoglycan epitope are present on endometrial MUC1.

Previous data showed that Mabs 5D4 and D9B1 both recognize sulfated lactosaminoglycan chains (Mehmet *et al.*, 1986; Hoadley *et al.*, 1990). In order to examine further the association with MUC1, metabolic labeling with [^{35}S]sulfate of secretory phase endometrium was carried out in short term explant cultures, and followed by immunoprecipitation. Extracts from labeled tissue contain MUC1 migrating as a single band as recognized by the anti-core protein Mab HMFG1 (Figure 2A, lane a), probably representing the presence in this specimen of two similarly sized alleles. Mab 5D4 precipitated a comigrating sulfated band (Figure 2A, lane c), indicating that MUC1 carries keratan sulfate chains. Mab D9B1 precipitated material with the corresponding mobility (Figure 2A, lane b), confirming that this epitope is also present on MUC1.

Conditioned culture medium was also used for immuno-precipitation analysis (Figure 2B). Mabs D9B1 and 5D4 each precipitated a band with identical mobility in the gel. This comigrated with the one seen in tissue extracts from the same experiment. The lower intensity of the band precipitated by D9B1 compared to the 5D4 band may reflect absence of the former epitope from a fraction of the secreted product. Mab HMFG1 failed to precipitate a detectable band from the culture medium (Figure 2B, lane a). HMFG1 binding affinity is lost from more highly glycosylated forms of MUC1, including secreted product seen immunohistochemically in gland lumens (Hey *et al.*, 1994). Thus, the product secreted in culture appears to lack this epitope. Even in the tissue-extracted form of the molecule, relatively little

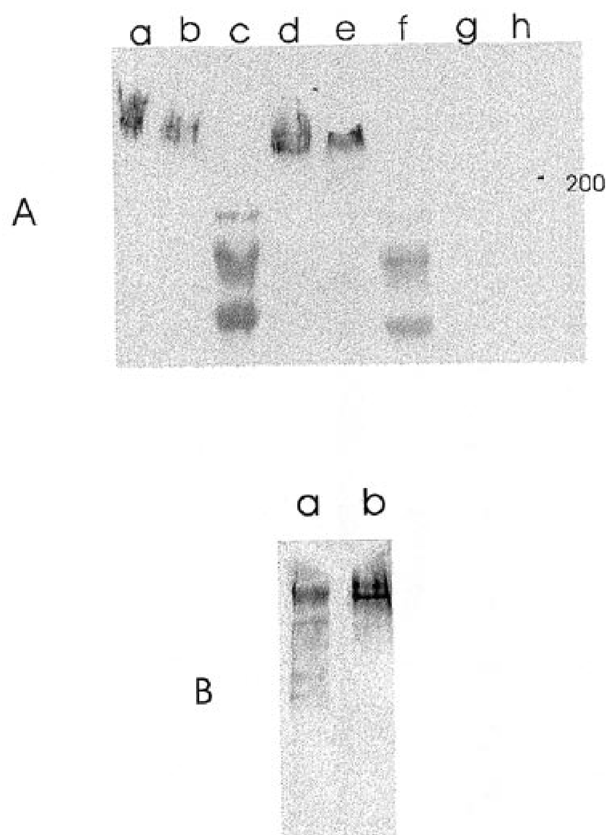

Fig. 1. Western blotting of endometrial tissue extracts with anti-MUC1 core protein and anti-glycan monoclonal antibodies. (**A**) Extracts of secretory phase tissue made in the presence (a, d, g) or absence (b, e, h) of protease inhibitors. Identically loaded lanes were probed with Mab D9B1 to a sialokeratan sulfate structure (a, b), Mab HMFG1 to MUC1 core protein (d, e) or control antibody (g, h) Lanes c and f contain prestained molecular mass standards (Sigma): phosphorylase b (97 kDa), β-galactosidase (116 kDa), and α2-macroglobulin (180 kDa). A comigrating doublet is apparent in lanes a, b, d, e. No proteolytic degradation is evident. (**B**) Secretory phase tissue extract probed with (a) Mab 5D4 to keratan sulfate or (b) Mab BC3 to MUC1 core protein showing comigrating species.

radioactive sulfate is present in the population of molecules precipitated by HMFG1 (Figure 2A, lane a). Western blots also indicate weak reactivity of HMFG1 with soluble MUC1 in tissue extracts (not shown).

Endometrium was cultured overnight, and then keratan sulfate-bearing glycoforms were immunoprecipitated with Mab 5D4, separated on urea-SDS–PAGE, and a blot prepared. This was probed with Mab BC3 to the core protein. A MUC1 immunoreactive doublet was detected at M_r > 250 kDa, confirming the finding that the mucin carries KS chains (Figure 3A, lanes a and c). Aliquots of the immunoprecipitate were treated with the enzyme endo-β-galactosidase. The enzyme-treated mucin, again detected by blotting with anti-core protein Mab BC3, migrated faster than the control molecule in urea-SDS–PAGE , indicating a reduction in its M_r (Figure 3A, lanes b and d). We estimate the cleaved carbohydrate to be in the 20-30 kDa range.

A similar experiment was carried out in which endometrial explant culture supernatants were treated with endo-β-galacto-sidase prior to immunoprecipitation with 5D4. This had the effect of reducing the amount of BC3-immunoreactive mucin in the

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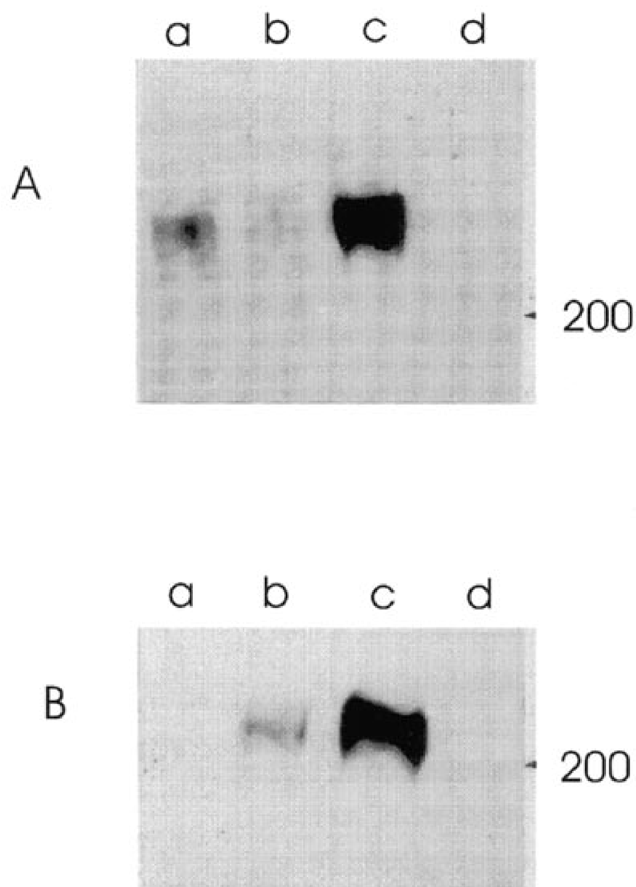

Fig. 2. Immunoprecipitation from secretory phase tissue after overnight culture in the presence of [^{35}S]sulfate. Results from tissue homogenate (**A**) are compared with the supernatant (**B**) Mabs: HMFG1 to core protein (a), D9B1 to mucin-associated sialoglycan (b), 5D4 to keratan sulfate (c) and a control (d). A single comigrating band is visible in each case, suggesting comigrating alleles in this individual. The data indicate that MUC1 (**A**, lane a) carries KS chains (**A**, lane c) with relatively little sialoepitope detected in the tissue extract (**A**, lane b). In secretions, sialylation is apparent (**B**, lane b) as is KS (**B**, lane c) but the core protein antibody immunoprecipitates the more heavily glycosylated secretory product inefficiently (**B**, lane a). The 200 kDa molecular weight marker is indicated.

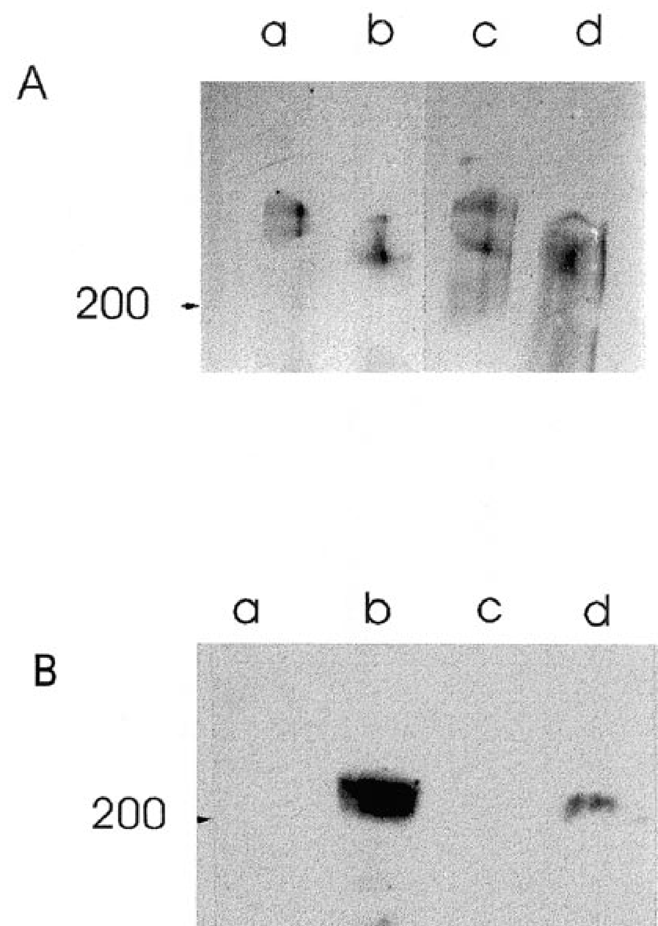

Fig. 3. (**A**) Secretory phase endometrium was cultured overnight and immunoprecipitates made using the anti-KS MAb 5D4 from the culture supernatant (lanes a and b) or tissue extract (lanes c and d) Aliquots of the precipitated material were treated with endo-β-galactosidase. After running out on urea-SDS–PAGE and transfer to a filter, the products were probed with MAb BC3 to the MUC1 core protein. Lanes a and c show control immunoprecipitates; lanes b and d show immunoprecipitates treated with endo-β-galactosidase. A doublet at M_r >250 kDa represents precipitated, KS-bearing MUC1. After enzyme treatment this shifts to higher mobility. (**B**) Secretory phase endometrium was cultured overnight and the supernatant collected. Aliquots were treated with endo-β-galactosidase then immunoprecipitated using Mab 5D4 or control antibody. The resulting blot was probed with Mab BC3. Lane a, control antibody, mock digested; lane b, 5D4, mock digested; lane c control antibody, enzyme digested; lane d, 5D4, enzyme digested. A smaller quantity of MUC1 is precipitated after enzyme treatment, indicating that KS chains on the mucin are cleaved by the enzyme, but some 5D4 epitopes remain. The 200 kDa molecular weight marker is indicated.

precipitate (Figure 3B), confirming that MUC1-associated lactosaminoglycan chains are partially sensitive to cleavage with endo-β-galactosidase.

Patterns of glycan expression vary in the luminal epithelium

We previously reported that MUC1 core protein is present in both proliferative and secretory phases of the cycle, with an approximately 6-fold increase in mRNA abundance in the early secretory phase, and a high level of transcript maintained through the mid secretory phase (Hey *et al.*, 1994). These observations reflect the composition of the glandular epithelial compartment of the functionalis, which is more abundant than luminal epithelium in curettings. Core protein abundance also increases in glands during the transition from proliferative to early secretory phase (Hey *et al.*, 1994). Since attachment of the blastocyst occurs at the luminal epithelium, there is a need to characterize this cell compartment in detail, especially in midsecretory phase when implantation occurs. The receptive phase is predicted to lie between days 20 and 24 of the normal cycle (Bergh and Navot, 1992). MUC1 core protein shows strong continuous staining in both glandular and luminal epithelium in the period between days 20 and 24 (Figure 4A,B). With Mab HMFG1, prior desialylation is required to reveal core protein epitopes in both glandular and luminal cells (Figure 4C,D).

In glandular epithelium the 5D4 epitope is regulated in a pattern that is essentially similar to the core protein, with low but detectable immunoreactivity in the proliferative phase, and a significant increase in the secretory phase when it appears in cells and secretions (Figure 5; Graham *et al.*, 1994). In the luminal epithelium, 5D4 shows strong continuous apical reactivity throughout the proliferative and early secretory phases (Figure

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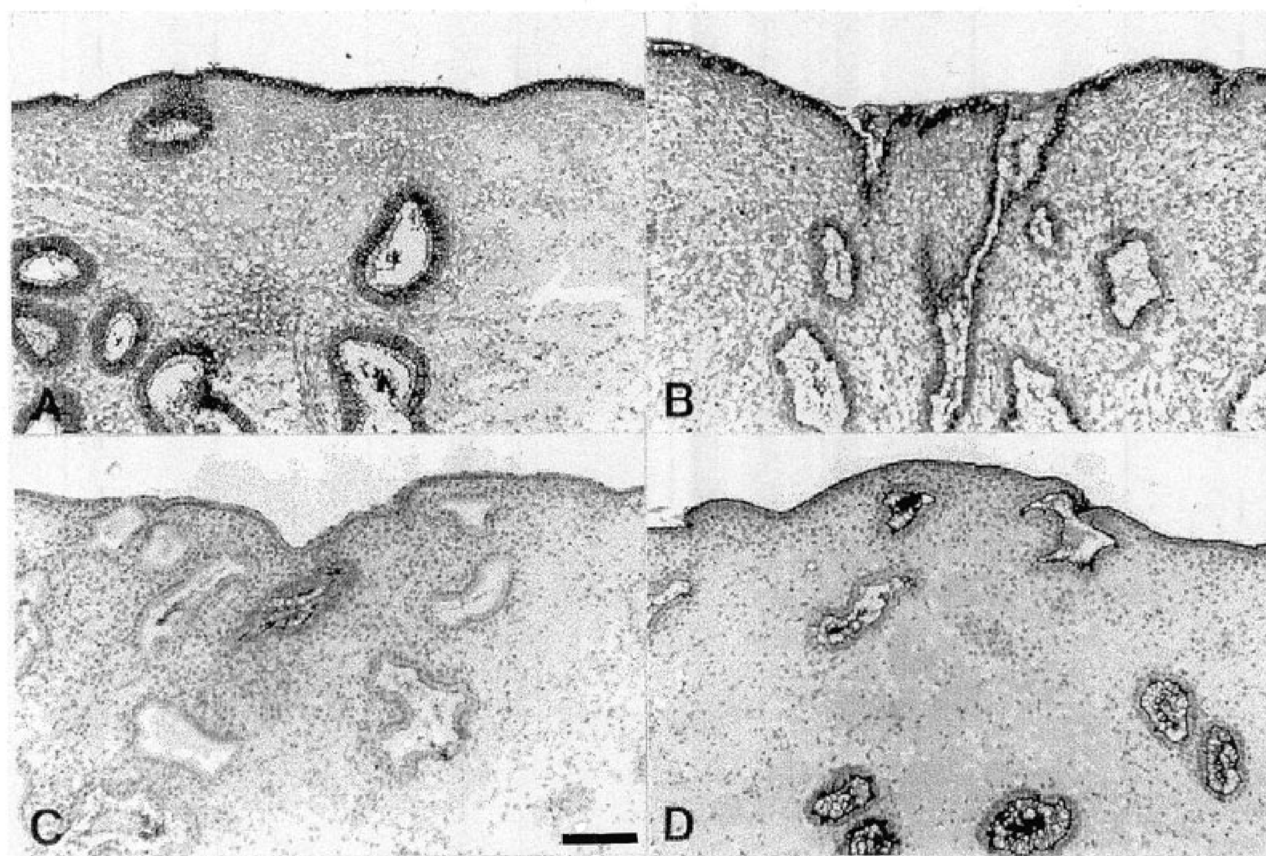

Fig. 4. MUC1 core protein staining in endometrial luminal epithelium with (**A**, **B**) Mab BC2, (**C**, **D**) Mab HMFG1. (**A**, **C**, **D**) Midsecretory phase; (**B**) late secretory phase. (**D**) Sialidase pretreated. The luminal reactivity is continuous in both mid and late secretory phases. Continuous linear apical HMFG1 binding is revealed only after desialylation. Scale bar, 100 µm.

5A,B). On day 20 apical staining is still evident in this compartment though there is some reduction in its intensity (Figures 5C, 6C,D). By day 24, some areas of the luminal epithelium lack immunoreactivity (Figure 6G,H). The superficial glands are also sometimes negative. In adjacent sections it is apparent that the same cells are still strongly positive for MUC1 core protein (Figure 6E,F).

The D9B1 epitope is absent from normal proliferative phase endometrium (Smith *et al.*, 1989). It appears in the secretory phase in gland cells, secretions, and the luminal epithelium (Figure 7A). However, staining is heterogeneous with some areas of glandular and luminal epithelium weak or negative (Figure 7B). Mab B72.3 recognizes the sialyl Tn epitope (NeuAc-α2–6GalNAc), commonly associated with mucin-type glycoproteins (Thor *et al.*, 1987; Hanisch *et al.*, 1996). B72.3 binding is also absent in proliferative phase, appearing in the secretory phase. As previously reported, heterogeneous staining is evident in secretory phase glandular epithelium (Figure 7C). Throughout the cycle, the luminal epithelium is uniformly negative for this epitope (Figure 7C).

Discussion

Neutral lactosaminoglycan chains have been shown to be associated with mammary MUC1 (Hanisch *et al.,* 1989, 1996), but to our knowledge this is the first demonstration of sulfated lactosaminoglycan (keratan sulfate) on MUC1. Both glycosylation and sulfation are likely to be tissue and cell type specific. Mab 5D4 recognizes a family of highly sulfated chains, the structure with highest binding affinity being an octasaccharide with one 6-sulfate at each residue (Mehmet *et al.*, 1985). Its strong binding to luminal epithelium suggests dense sulfation of MUC1, indicating that the apical luminal epithelium carries a highly electronegative glycocalyx, and this may contribute to electrostatic repulsion of the embryo in stages of the cycle that precede receptivity. Since no binding of B72.3 (sialyl Tn) occurs at the luminal epithelium, the luminal phenotype may be characterized by longer glycans and, hence, a more complex pattern of MUC1 glycosylation. If so, it is reasonable to postulate that the glycocalyx may be involved in the control of receptivity, as well as in other functions of this epithelium such as providing a barrier to pathogenic attack on the upper genital tract. Despite their common origin during postmenstrual regeneration, the luminal epithelium exhibits a differentiation state that is distinct from that of the glandular epithelium.

The D9B1 epitope can be abolished from endometrium either by sialidase, keratanase, or endo-β-galactosidase treatment, results that show the epitope includes a terminal sialic acid residue associated with sulfated lactosaminoglycan chains (Hoadley *et al.*, 1990). 5D4 binding to endometrial MUC1 is reduced, but not abolished, by endo-β-galactosidase treatment. The precise substrate specificity of the *B.fragilis* enzyme has not been reported, but closely related enzymes cleave at C1 of unbranched and unsulfated galactose residues (Maley *et al.*,

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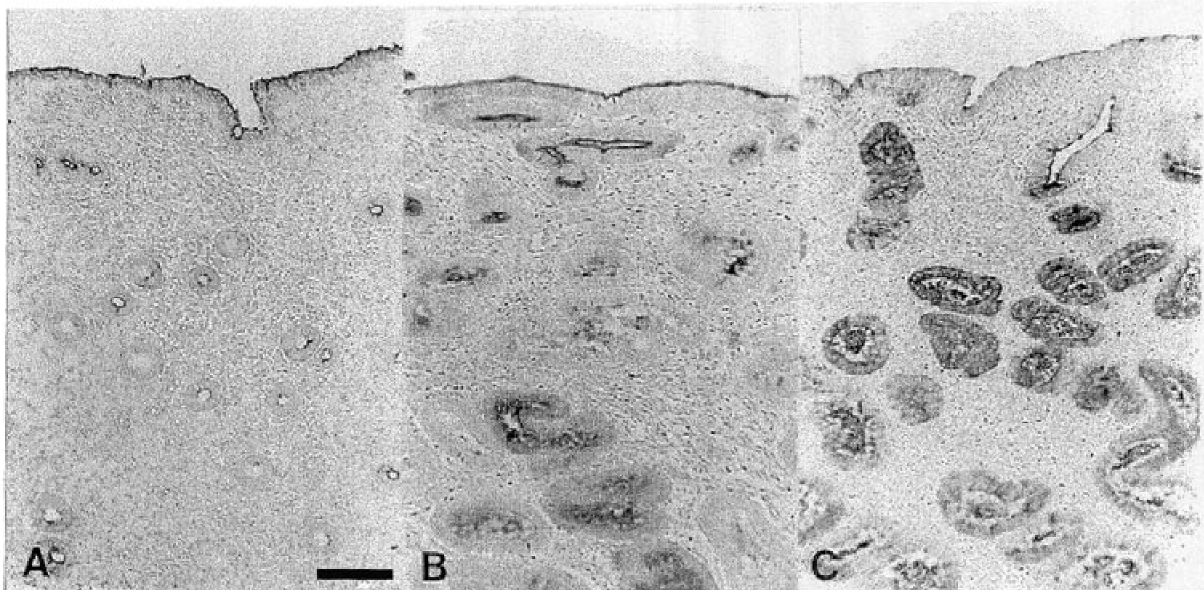

Fig. 5. Mab 5D4 stains the endometrial luminal epithelial apical surface strongly in proliferative (**A**), early secretory (**B**), and midsecretory phases (**C**). However, a reduction in staining intensity is apparent in midsecretory phase. Scale bar, 140 μm.

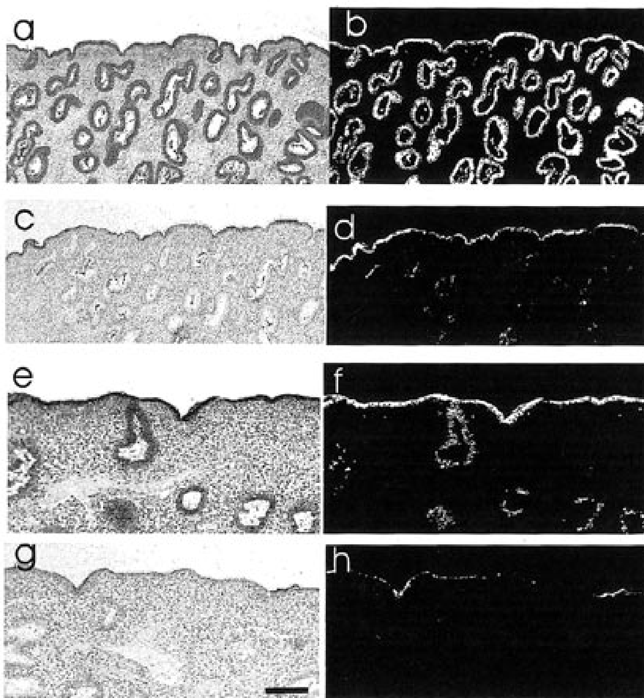

Fig. 6. MUC1 core protein (Mab BC2; a, b, e, f) and keratan sulfate (Mab 5D4; c, d, g, h) in adjacent sections from postovulatory day 6–7 (a–d) or 10–11 (e–h) Images b, d, f, and h have been segmented electronically to show the immunostain as white. Note the continuous reactivity of the luminal apical epithelium for core protein on both days. Keratan sulfate is continuous in this location at day 6–7, but staining is interrupted by day 10–11, discrete patches of unstained epithelium becoming apparent. Scale bar, 100 μm.

1984), so not all residues may be sulfated. On the other hand, the incomplete abolition of 5D4 sites suggests a possible occurrence of densely sulfated regions of glycan chain close to the attachment to core protein. It is not yet known whether the loss of 20–30 kDa after endo-β-galactosidase treatment is made up of one or several chain cleavage events.

The vast majority of glycan substituents are likely to be O-linked to residues within the VNTR or its serine/threonine-rich flanking sequences. However, the membrane-proximal domain of MUC1 contains several potential N-glycan attachment sites (Gendler *et al.*, 1990). N-Glycanase treatment does not affect binding of 5D4 (data not shown), which might suggest O-linkage to core protein, but further work is required to define the sites of attachment of these sulfated oligosaccharides. Most information pertaining to MUC1 glycosylation has been derived indirectly through the observation that many Mabs which bind the VNTR do so through a highly immunoreactive sequence PDTRP. Glycosylation of flanking T (and S) residues occurs, causing steric inhibition of antibody binding (Stadie *et al.*, 1995). This is borne out in endometrium (including both luminal and glandular compartments) by the finding that sialidase pretreatment increases the binding of Mab HMFG1 (Hey *et al.*, 1994; this study). The sialylated, sulfated chains recognized by Mab D9B1 are present in the luminal epithelium, though their distribution is uneven. Thus, despite the absence of sialyl Tn, the luminal epithelial surface contains sialylated as well as sulfated glycan structures.

Biochemical evidence in the current study confirms that endometrial MUC1 consists of an envelope of glycoforms—for example, not all 5D4-binding MUC1 also binds D9B1. Immunoprecipitation demonstrates that tissue-associated MUC1 retains more HMFG1 binding sites, and that the secreted form binds more D9B1 and less HMFG1. Evidence for the presence of both cell surface and secreted forms of the molecule as well as the molecular basis for release from cells has been discussed previously (Aplin and Hey, 1995). The data are consistent with higher levels of glycan on the released pool and the presence of intracellular reserves of incompletely glycosylated product,

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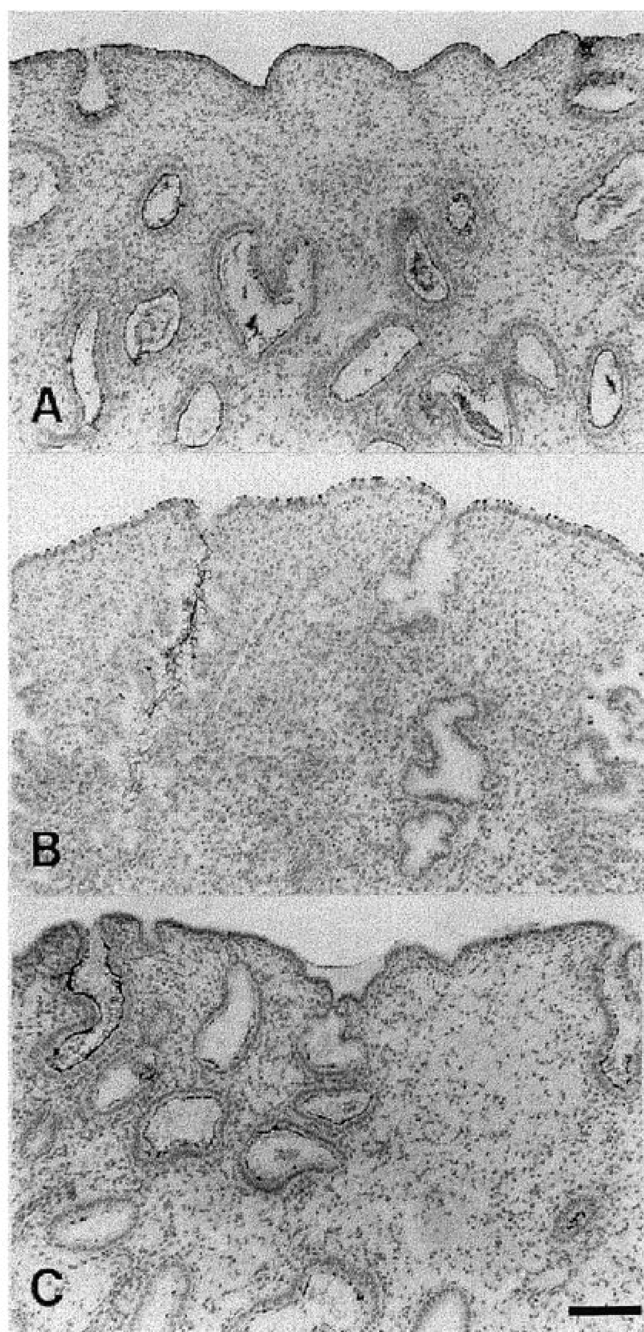

Fig. 7. Sialoglycan epitopes recognized by Mabs D9B1 (**A**, **B**) and B72.3 (**C**) in mid secretory endometrium. D9B1 staining is present in both glandular and luminal epithelial cells but there is heterogeneity in both populations. Luminal reactivity is continuous in some areas (**A**) but punctate in others (**B**). Luminal epithelium lacks immunoreactivity for antibody B72.3, which is confined to the glandular compartment (**C**). Scale bar, 100 µm.

presumably in the process of biosynthesis. The strong upregulation of several sulfo- and sialoglycans in secretory phase is consistent with the postovulatory increase of Golgi stacks (Dockery *et al.*, 1988). It is probable that sialyl and sulfotransferase activities increase as a result of progestrogenic stimulation, but this will have to be examined more directly. Histochemical investigations using the lectin DSA (datum stramonium agglutinin) suggest that core lactosaminoglycan is present in epithelial cells throughout the cycle, so that the later, more distal glycan modifications such as sulfation and sialylation may be the most sensitive to hormonal effects. Core protein production is itself affected by hormone (Hey *et al.*, 1994), so the net production of glycan presumably depends on both availability of core protein and the presence of appropriate transferases. Normal endometrium is inherently heterogeneous in relation to the production of glycans—this is evident in the immunohistochemical comparison of gland profiles when a variety of degrees of reactivity are always evident (see, e.g., Thor *et al.*, 1987; Graham *et al.* , 1994). Thus, while glycan fingerprinting may be useful as an index of differentiation or hormone effects, care must be used to achieve a representative tissue sample. Curettings or hysterectomy specimens are preferable to biopsies in this regard.

The continuous apical staining we have observed with antibodies to MUC1 core protein in the luminal epithelium throughout the endometrial cycle in women precludes a pattern of behavior analogous to the mouse (Braga and Gendler, 1993) and pig (Bowen *et al.*, 1996), where MUC1 is lost from the cell surface prior to implantation. This is consistent with MUC1 contributing to the maintenance of an anti-adhesive, nonreceptive luminal surface in the prereceptive phase, with disappearance to (presumably) unmask proadhesive components at implantation. In baboon, reduction of cell surface MUC1 has been observed in the implantation phase, but it is not absent (Hild-Petito *et al.* 1996). In women, our data suggest subtle alterations in the glycoform envelope in the luminal epithelium, with the appearance during the predicted implantation phase of areas that contain lower degrees of sulfation (less 5D4 binding). This may reduce Coulombic repulsion between the negatively charged trophectoderm and the maternal epithelium in selected areas. It is also the case that specific isoforms of MUC1 appear that carry recognition motifs such as sialyl Lewis x (Hey and Aplin, 1996), and this mechanism could target the embryo to specific sites for implantation. Lastly, information obtained in nonconception cycles is by definition incomplete. It is entirely possible that specific microdomains of structural modulation occur as a result of short range maternal–embryo communication.

Materials and methods

Tissue

Bouin's fixed, wax-embedded endometrial tissue was obtained at dilatation and curettage or hysterectomy. Dating was based on the time of the last menstrual period and later confirmed histologically according to the method of Noyes *et al.* (1950). Data from specimens not in phase or showing pathological features including inflammation or neoplasia were excluded retrospectively from the results. The data are based on 40 normal specimens distributed across the cycle.

Antibodies

Anti-keratan sulfate Mab 5D4 (Mehmet *et al.*, 1986; Funderburgh *et al.*, 1987) was purchased from ICN Biomedical (Thame, Oxfordshire, UK). Mab D9B1 was raised to endometrial epithelial glands as described previously (Graham *et al.*, 1989; Hoadley *et al.*, 1990). Mab B72.3 recognizes sialyl Tn (Thor *et al.*, 1988; Hanisch *et al.*, 1996). Mabs to MUC1 all bound to sequences overlapping the peptide PDTRP which forms part of the tandem repeat sequence in the ectodomain. They were as follows: BC2 and BC3 (Xing *et al.*, 1989; Hey *et al.*, 1995); HMFG1 and HMFG2 (Burchell *et al.*, 1987; Hey *et al.*, 1994).

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Mouse immunoglobulins were used as control antibodies in immunochemical protocols.

Immunochemical procedures

Tissue was snap-frozen in liquid nitrogen and stored at -80°C. For sample preparation the tissue was defrosted on ice. One milliliter of cold 0.05 M Tris–HCl (pH 7.6) was added per 100 mg tissue. Tissue was homogenized using a polytron and incubated at 4°C for 1 h. After centrifuging for 1 min at $13 \times g$, the supernatant was collected and dialyzed against distilled water overnight at 4°C. The sample was freeze dried.

Endometrial explant culture

Tissue was collected into Eagle's Minimal Essential Medium (MEM; Gibco, Paisley, UK) containing 25 mM HEPES, penicillin, and glutamine. After washing thoroughly to remove blood, the tissue was cut into pieces of approximately 1–2 mm diameter and ~100 mg was preincubated for 1 h in 1 ml culture medium at 37°C in 95% air, 5% CO_2. It was then incubated for 18 h in 100 µCi/ml of sodium [^{35}S]sulfate. Medium and tissue were processed separately.

Immunoprecipitation

Spent culture medium or an extract of tissue homogenate was precleared with unloaded protein A–Sepharose (50 µl packed beads, diluted 1:1 in fresh culture medium) for 1 h at 4°C, and the supernatants were aliquotted. Mab was added as follows: HMFG1 150 µl/ml; D9B1 150 µl/ml; 5D4 8 µl/ml. Incubation proceeded for 2 h at room temperature. Protein A–Sepharose beads, diluted 1:1 with TBS (20 µl per precipitation reaction) were preloaded for 17 h at 4°C to 1/20th of their capacity with anti-mouse IgG (8 µg/µl, 25 µl; Dako) and then incubated for a further 2 h at room temperature. Beads were pelletted and washed six times in TBS and finally resuspended 1:1 in reducing sample buffer (125 mM Tris/HCl, pH 6.7 containing 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, and 0.04% (w/v) bromophenol blue). After boiling for 10 min, 8 M urea was added to the samples which were incubated at room temperature for 17 h before running on an 8 M urea/SDS/5% polyacrylamide gel. Gels were fixed in destain for 1 h, soaked in glacial acetic acid (2×1 h) and immersed in 22% (w/v) 2,5-diphenyloxazole (PPO, BDH) in glacial acetic acid for 3 h. The fluorophore was precipitated *in situ* by washing the gel in water for 1 h. Finally, the gel was dried onto Whatman No. 3 filter paper and exposed to preflashed Kodak XR5 x-ray film. Films were exposed at -80°C.

For immunoprecipitation followed by Western blotting, spent culture medium (nonradioactive) and tissue homogenate samples were divided into two equal aliquots. Immunoprecipitation was performed with Mab 5D4 or control antibody, and filter transfers were made to nitrocellulose.

For glycosidase digestion, 5D4 immunoprecipitates were collected on protein A beads as described above. After washing the beads six times in TBS they were resuspended in 45 µl 50 mM sodium acetate buffer, pH 5.8, and boiled for 10 min. After cooling, 5 µl endo-β-galactosidase (*Bacteriodes fragilis*, 125 mU/ml protease free; Oxford Glycosystems) was added, and the solution was incubated for 17 h at 37°C before processing for urea/SDS–PAGE and transfer to nitrocellulose. In an alternative protocol, tissue extract or explant culture supernatant was treated with endo-β-galactosidase prior to the immunoprecipitation.

To detect core protein in the precipitates, membranes were probed with Mab BC3 using enhanced chemiluminescent detection. Membranes were blocked for 1 h at room temperature or for 17 h at 4°C in Blotto (0.15 M NaCl, 0.05% (v/v) Tween 20, 3% (w/v) milk solids, 0.02% (w/v) sodium azide, 50 mM TrisHCl, pH 7.6) and then incubated in BC3 (1/40 in Blotto) on a shaker for 17 h at room temperature. After 3×5 min washes in Blotto they were incubated in peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako, 1/50) for 1 h at room temperature and washed 3×10 min in TBS containing 0.1% Tween 20 followed by 5 min in distilled water. Antibody binding was detected as described by the manufacturer (Amersham Life Sciences, Amersham, UK). Filters were wrapped in cling film and exposed to Kodak XR5 for 30 sec to 2 min.

Immunohistochemistry

Tissue was immediately fixed in Bouin's solution or formaldehyde (10% phosphate-buffered formalin) for 2–4 h at room temperature and embedded in paraffin wax. Endogenous peroxidase activity was blocked by treating sections for 30 min in H_2O_2/methanol (1/100 v/v). After washing in distilled water for 5 min, staining was carried out as described previously (Hey *et al.*, 1994; Hey and Aplin, 1996).

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