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MUC1 is a scaffold for selectin ligands in the human uterus

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1. ABSTRACT

MUC1 is a large, transmembrane mucin glycoprotein abundantly expressed at the apical surface of uterine epithelia in all species examined to date. Loss of MUC1 at the time of embryo implantation occurs in many species; however, this does not appear to be the case in humans. Recent studies indicate that human blastocysts express L-selectin at their external surfaces raising the possibility that selectin ligands expressed at the apical surface of the uterine epithelium support early stages of blastocyst attachment. In the current study, we have used a panel of antibodies specific for selectin ligands to determine if MUC1 functions as a scaffold for these carbohydrate motifs in fertile women. The results demonstrate that MUC1 carries selectin ligands throughout the secretory phase of the menstrual cycle, including the mid-secretory (receptive) phase. Consequently, MUC1 represents a potential ligand for selectins expressed by human blastocysts.

2. INTRODUCTION

A critical early event in embryo implantation is the attachment of the external surface of the blastocyst trophectoderm to the apical surface of uterine epithelia. Several cell adhesion molecules supporting firm attachment have been identified at these sites in various species, notably integrins, integrin ligands, heparan sulfate proteoglycans and their binding proteins (1). In many, if not all, cell adhesion events the initial stages of attachment involve weaker, "tethering" interactions involving protein-carbohydrate binding followed by stronger protein-protein binding events (2, 3). In the former case, interactions of mammalian lectins, most notably selectins, are implicated with specially modified carbohydrates simply referred to as selectin ligands serving as their partners. Selectin-dependent binding typically requires fluid shear force to be effective (2). While it is unlikely that the uterine lumen ever encounters the level of fluid shear force found in the blood stream, it is not clear what level of shear force occurs

in this environment or if this varies during the cycle. Recent studies have demonstrated that human blastocysts express L-selectin on their external surfaces (4) raising the possibility that these molecules may participate in early stages of human blastocyst attachment. Furthermore, the lack of detection of L-selectin at earlier embryonic stages indicates that this cell surface protein is developmentally regulated (5). Nonetheless, complementary carriers or scaffold proteins bearing selectin ligands on the apical surface of uterine epithelium have not been identified.

MUC1 is a transmembrane mucin glycoprotein abundantly expressed at the apical surface of uterine epithelia in all species examined to date (6). While MUC1 is lost at the uterine luminal surface prior to blastocyst attachment in many species, this does not appear to be the case in humans. In fact, MUC1 not only persists, but also appears to undergo carbohydrate modifications during the mid-luteal (receptive) phase in humans suggesting a positive association with the implantation process in this species (7). The large external domain of MUC1 consists primarily of a series of 50 -150 tandem repeats of 20 amino acids highly enriched in serine, threonine and proline residues (reviewed in (8). In humans, the variable number of tandem repeat motifs results in allelic polymorphism that generates both mRNA and mature MUC1 glycoproteins of different sizes. These tandem repeats are heavily substituted with O-linked (mucin-type) oligosaccharides. The net effect is to produce an external domain that extends several hundred nanometers from the cell surface, i.e., far beyond the distance spanned by typical cell surface proteins, e.g., integrins (9). Consequently, if MUC1 persists at the time of embryo attachment, it is highly likely that this is one of the first, integral uterine cell surface components encountered by the attaching blastocyst.

The current study was performed to determine if human uterine MUC1 carries selectin ligands and, thereby, potentially serves as a candidate binding partner for L-selectins expressed by the human blastocyst. Using a panel of antibody probes, we demonstrate that MUC1 is a scaffold for selectin ligands throughout the secretory phase of the cycle, including the mid-secretory (receptive) phase. Consequently, MUC1 may facilitate early aspects of embryo attachment in humans.

3. MATERIALS AND METHODS

3.1. Endometrial samples

Endometrial biopsies were obtained from normal women with proven fertility either in their proliferative phase or after documenting a urinary LH surge as described (10). The samples from different times in the secretory phase were histologically "in phase" based on the criteria of Noyes *et al.* (11). Tissue collection was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill. Biopsies were homogenized on ice using RIPA extraction buffer (10 mm Tris HCl, pH 8.0; 10 mm EDTA, pH 8.0; 0.15 m NaCl; 1% [v/v] NP-40; 0.5% [w/v] SDS) containing a protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN). After centrifugation at 14,000 rpm/10 min, protein concentrations were determined in the supernatant using the Bio-Rad Laboratories, Inc. (Hercules, CA) protein assay kit and lysates were stored at -80° C until used.

3.2. Antibodies

The following antibodies were used for these studies. The mouse monoclonal antibody, 214D4 (12), reacts with the glycosylated MUC1 ectodomain with reactivity that is minimally affected by the MUC1 glycoform. MECA-79, CSLEX-1, HECA-452 and anti-PEN5 are previously described mouse or rat monoclonal antibodies with specificities toward selectin carbohydrate ligands (13-17). HECA-452 recognizes sLe^x and related structures including 6-sulfo-sLe^x, 6'-sulfo-sLe^x and 6,6'-disulfo-sLe^x. The MECA-79 antibody recognizes a high-affinity L-selectin ligand carbohydrate epitope containing 6-sulfo-N-acetylglucosamine. Additional information on the respective reactivities of the selectin ligand antibodies is indicated in Figure 3.

3.3. Immunoprecipitation and Western blotting

Endometrial extracts (100 µl) prepared in RIPA buffer were diluted tenfold in 0.5% (v/v) Nonidet P-40 in PBS and clarified by centrifugation. Equal volumes (500 µl) of diluted lysates were incubated with 40 µl mouse monoclonal antibody 214D4 (256 ng IgG_1) or isotype matched nonimmune mouse IgG overnight at 4°C. Antigen-antibody complexes were incubated overnight at 4°C with constant agitation after addition of 50µl of a 50% (v/v) slurry of protein G-Sepharose (Sigma) that had been previously blocked with fetal bovine serum. The pelleted resin was washed twice with 500 µl of 0.5% (v/v) NP-40 in PBS and twice with PBS. Resin pellets were extracted by boiling 2 min in 50 µl sample extraction buffer and 50 µl Laemmli sample buffer (18) , followed by centrifugation prior to application to the gel. Sample extraction buffer consisted of 0.05 M Tris, pH 7.0, 8 M urea, 1.0% (w/v) SDS, 1.0% (v/v) β-mercaptoethanol, and 0.01% (w/v) phenylmethylsulfonyl fluoride. Samples (20 µl) were resolved on 10% (w/v) SDS-polyacrylamide gels (19), transferred to nitrocellulose in 100 mM Tris base and 100 mM glycine, pH 8.3, and blocked for 4 h at 4°C in 3% (w/v) BSA (fraction V, Sigma), 0.1% (v/v) Tween 20 in PBS. Primary antibodies diluted in blocking solution were incubated with the blots overnight at 4°C, rinsed three times with 0.1 % (v/v) Tween 20 in PBS, and incubated 4 h at 4°C with the appropriate horseradish peroxidase conjugated second antibody diluted 1-200,000 in blocking solution. Blots were rinsed three times for 5 min in 0.1 % (v/v) Tween 20 in PBS. Antibody was visualized by ECL reagents applied according to the manufacturer's direction (Pierce). For antibodies 214D4 (mouse monoclonal, IgG_1), anti-PEN5 (mouse monoclonal IgM from Immunotech), and CSLEX-1 (mouse monoclonal IgM), the secondary antibody was`HRP-conjugated sheep anti-mouse IgG or HRP-conjugated anti-mouse IgM (Jackson Immunologicals). Primary antibodies HECA-452 and MECA-79 (both rat monoclonal IgM from Pharmigen) were detected by HRP-conjugated goat anti- rat IgM (Jackson Immunologicals).

4. RESULTS

Human endometrial extracts were solubilized and immunoprecipitated with a broadly reactive antibody

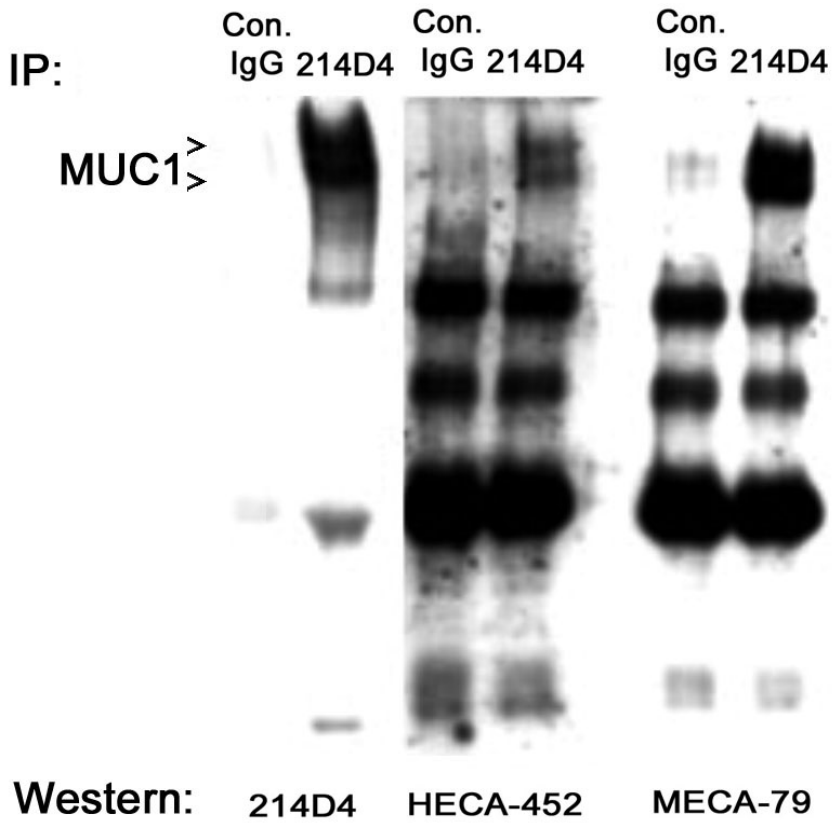

Figure 1. Specificity of MUC1 immunoprecipitation. A human endometrial extract from d. 22 of the cycle (day 7 post ovulation) was immunoprecipitated with 214D4 antibody (214D4) or control mouse IgG (Con. IgG) and subsequently analyzed by SDS-PAGE and Western blotting with the antibodies indicated at the bottom of the figure as described in Materials and Methods. The migration positions of the expected products of the two MUC1 alleles are indicated by the carets to the left of the figure. These bands are observed in the 214D4, but not the control IgG, immunoprecipitates. Additional, faster migrating bands reflect the reactivity of the secondary HRP-conjugated anti-mouse or rat immunoglobulin antibodies with 214D4 or mouse IgG used in the immunoprecipitations and are of similar intensity in both cases.

against MUC1 ectodomain as described (13). In our initial experiments, we determined the specificity of the immunoprecipitations by using appropriate non-immune control antisera. As shown in Figure 1, the MUC1-specific antibody, 214D4, immunoprecipitated material migrating at the expected large size and reacted with the 214D4 antibody in a Western blot. Bands of similar size were detected in the 214D4, but not nonimmune Ig, immunoprecipitates using the selectin ligand directed antibodies, HECA-52 and MECA-79. No specific MUC1 reactivity was observed using the PEN5 antibody which was not used in further studies (data not shown). Collectively, it appeared that the immunoprecipitation was specific and would serve as a valid method of detecting selectin ligand association with MUC1 in human endometrial extracts.

In the next experiment, a series of endometrial samples were obtained from fertile women at various stages of the menstrual cycle. MUC1 was immunoprecipitated with the 214D4 antibody or control mouse IgG and these immunoprecipitates subsequently were probed with a library of antibodies directed against selectin ligands. As shown in Figure 2, little 214D4 reactive MUC1 was detected during the proliferative stage compared to the luteal phase samples. In most cases, two major bands were detected consistent with the allelic polymorphism of this gene (8). Two human uterine epithelial cell lines, HES and Ishikawa, also were included in these assays. HES robustly expressed MUC1 while Ishikawa cells displayed very low levels and effectively served as negative controls. MECA-79 reactivity paralleled 214D4 reactivity in all endometrial extracts. In contrast, HECA-452 and CSLEX-1 reactivity were quite different from MECA-79, but paralleled each other. These antibodies displayed strong reactivity with MUC1 isolated from one early and one late secretory phase patient, but displayed modest reactivity with MUC1 from other samples. anti-PEN5 did not react with samples at any stage (data not shown). Parallel blots of the control IgG immunoprecipitates were negative with all antibodies for bands at the MUC1 positions, but displayed a similar profile of non-specific recognition of immunoglobulins present from the immunoprecipitation (not shown). MUC1 derived from HES cells failed to react with any of the selectin ligand antibodies. Additional experiments were performed in which HES cells were pretreated with

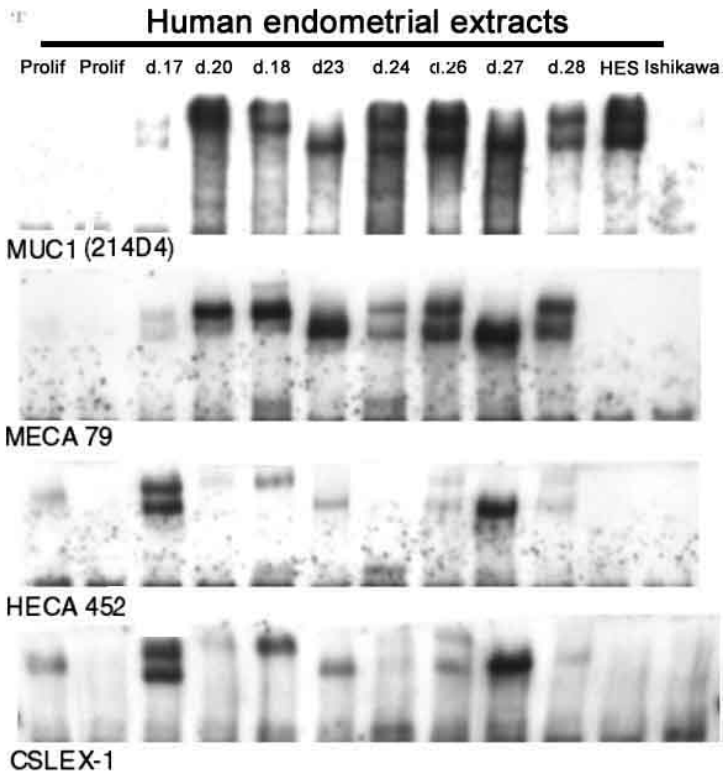

Figure 2. MUC1 reactivity with selectin ligand antibodies throughout the menstrual cycle. Immunoprecipitation with the MUC1-directed antibody, 214D4, and Western blotting with the indicated antibodies was performed as described in Materials and Methods. Similar extracts from human uterine epithelial cell lines expressing very high (HES) and very low (Ishikawa) levels of MUC1 were used as positive and negative controls as well. Endometrial staging was performed as described (10, 11). Note that MECA-79 reactivity closely parallels that of the 214D4 (total MUC1) reactivity while CSLEX-1 and HECA-452 immunoreactivity both display considerable variability relative to 214D4, but closely parallel each other. MUC1 isolated from either HES or lshikawa cells displayed no reactivity with any of the selectin ligand-directed antibodies.

combinations of interferon-γ and TNF-α before MUC1 isolation; however, these treatments did not result in accumulation of selectin ligands even in whole cell extracts (data not shown). Collectively, these data indicate that MUC1 carries selectin ligands throughout the secretory phase in fertile women. In addition, the differences in reactivity observed between MECA-79 *versus* HECA-452 and CSLEX-1 suggest that expression of selectin ligand glycoforms either differ among women or change during the secretory phase.

5. DISCUSSION

The current study demonstrates that human endometrial MUC1 carries selectin ligands during the secretory phase of the menstrual cycle. Little MUC1 was detected in uterine extracts from the proliferative phase, although previous studies indicate that MUC1 is expressed at this phase (7, 20, 21). This study also indicates that MUC1 carbohydrate structures undergo sulfation which result in glycoform alterations throughout the menstrual cycle. Therefore, our failure to immunoprecipitate MUC1 from the proliferative phase samples may reflect: 1) poor reactivity with the 214D4 antibody at this stage; 2) lower proportion of epithelial cells within the mix of cells in endometrial samples at this stage of the cycle; 3) lower expression levels of MUC1 at this stage, or; 4) a combination of these three possibilities. Of a wide variety of human MUC1 ectodomain antibodies we have examined, we have found the 214D4 antibody to be the least sensitive to glycoform differences and reliably displays strong reactivity in cells expressing significant MUC1 (J. Julian and D.D. Carson, unpublished observations). Nonetheless, we have observed that cytokine-stimulated HES cells express forms of MUC1 reactive with the HMFG-1, but not 214D4 antibody (P. Wang and D.D. Carson, unpublished observations). Due to the limited amount of material available for the current studies, we were unable to perform additional immunoprecipitations with other MUC1 directed antibodies to determine if MUC1 also carries selectin ligands during the proliferative phase.

Several previous studies demonstrated the occurrence of selectin ligands in human endometrium, including luminal epithelium, during the menstrual cycle. Hey and Aplin (21) reported that expression of the low affinity selectin ligands, i.e., sialyl Lewis x and sialyl Lewis a, generally paralleled that of MUC1, but did not identify the carriers of these oligosaccharides. MUC1

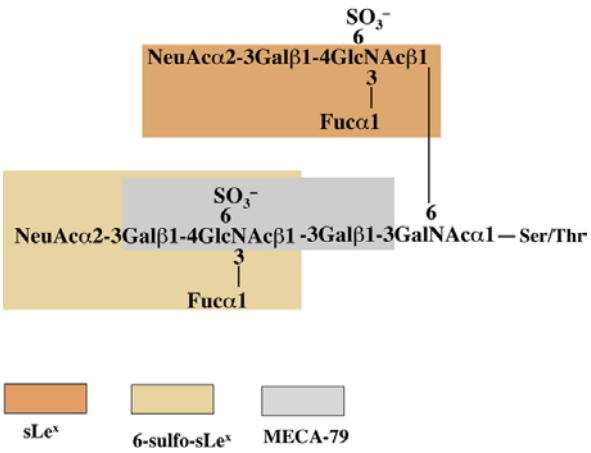

Figure 3. Epitopes recognized by selectin ligand-directed antibodies. The figure shows a hypothetical mucin-type oligosaccharide bearing multiple motifs recognized by the various antibodies used in the current study (13-16). Ser/Thr represents the protein core attachment site. The tan box indicates a sulfated modification of this motif, 6-sulfo-sLe^x. The HECA-452 antibody recognizes sLe^x, 6-sulfo-sLe^x and other related structures while the CSLEX-1 antibody recognizes sLe^x and related structures, but not 6-sulfo-sLe^x or 6,6'-disulfo-sLe. The grey box indicates a minimal structure required to be recognized by the MECA-79 antibody. Abbreviations: Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; NeuAc, N-acetylneuraminic acid; $S0_3$, sulfate.

levels are substantially lower in proliferative stage uteri and, thus, these observations qualitatively agree with the present studies. Yamaguchi *et al.* (22) also reported staining of sialyl Lewis a and 6-sulfo-sialyl Lewis x in mid-secretory stage samples that paralleled staining of proposed carriers; however, they also did not identify the proteins carrying these motifs. Lai *et al.* (23) also reported that L-selectin ligand reactivity in the uterine epithelium was greatest during the early to mid secretory phase using the MECA-79 antibody as probe. Genbacev *et al.* (4) stated that proliferative (follicular) stage samples reacted weakly with selectin ligand antibodies, whereas staining markedly increased in secretory stage samples at the apical epithelial surface. In their studies, MECA-79 appeared to consistently and robustly stain secretory phase uterine epithelia; however, they also reported that HECA-452 only stained 3 out of 6 samples tested. In the current studies, HECA-452 reacted strongly with MUC1 in 2 out of the 8 samples while MECA-79 strongly reacted with 7 out of 8. Thus, expression of the MECA-79 epitope appears to be a more reliable marker of the appearance of high affinity selectin ligands in this tissue. Compared to MECA-79, HECA-452 and CSLEX1 require the presence of an additional fucose residue for reactivity (Figure 3); (12, 13). The similar staining patterns observed with these two antibodies may be due to the broader range of reactivity of HECA-452 including CSLEX-1 reactive epitopes. Thus, it is possible that variability in fucosylation accounts for the differences among these individuals.

Overall, our observations are in good agreement with the previous studies of selectin ligand expression in human endometrium and identify MUC1 as one of the selectin ligand scaffolds. This raises the possibility that MUC1 might mediate early, selectin-dependent attachment reactions of the human blastocyst with the uterine surface, a suggestion in marked contrast with the antiadhesive role proposed for MUC1 in other systems (1, 6). If selectin ligand-bearing MUC1 supports initial human blastocyst attachment, this function does not appear to track strictly to the receptive state since MUC1 carries these ligands throughout the secretory phase. Lymphocyte populations change dynamically in human endometrium during pregnancy and cycle in humans and other species (24-26). Thus, it is possible that selectin ligands play a constitutive role in lymphocyte transit to the uterine lumen to confront microbial challenges. In the context of human blastocyst attachment, *in vitro* studies indicate that MUC1 is likely to be removed at the site of attachment (27). This would provide access to other cell surface receptors, e.g., integrins, that generally provide stronger binding sites. In this regard, MUC1 "sheddases" have been identified in human uterine epithelia that could support such MUC1 clearing at embryo attachment attachment sites (28, 29). The observation that MUC1 sheddase systems are stimulated by proinflammatory cytokines (30) provides further support for the idea that many of these events may be primarily associated with mucosal defense and secondarily adapted for the implantation reaction.

In summary, we have shown that MUC1 is one of the carriers of selectin ligands in human endometrium. It remains to be determined if blastocysts use selectins to interact with MUC1 and other selectin ligand carriers during early stages of the human implantation process or if MUC1 carries selectin ligands in uteri of other species. Finally, since effective selectin-selectin ligand tethering requires fluid shear stress, it is of interest to determine what shear forces exist in the uterine lumen and if this changes during the transition to the receptive state.

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