

THE GLYCOBIOLOGY OF IMPLANTATION

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1. ABSTRACT

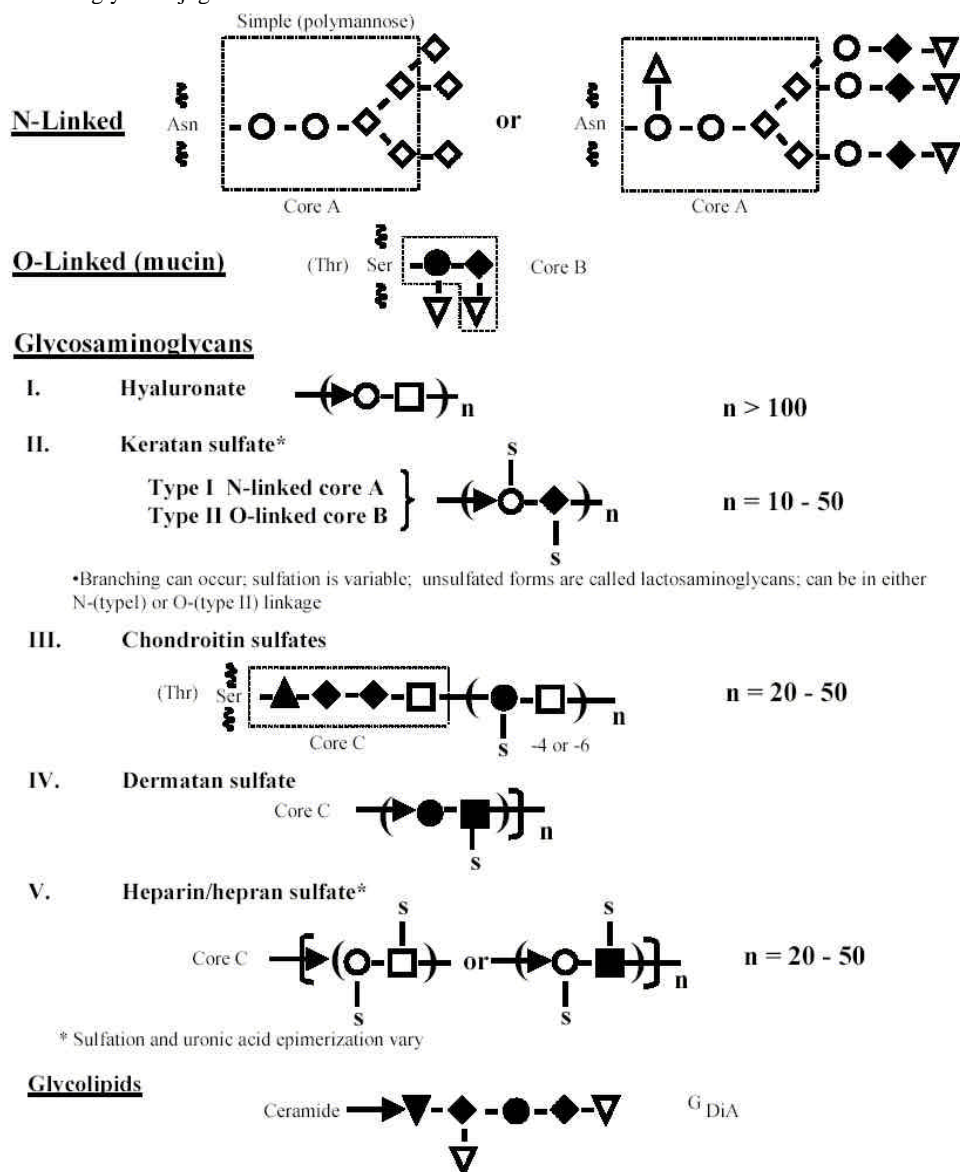
Embryo implantation involves complex, coordinated interactions of cell surface components of embryonic (trophectoderm/trophoblast) and uterine cells. Oligosaccharides are common constituents of cell surface molecules. A variety of oligosaccharide structures have been identified at embryonic and uterine cell surfaces and change dynamically during progression of the implantation reaction. In many cases, the oligosaccharides have been shown to have biological activities with great relevance to the implantation process. This review summarizes the available information on glycoconjugates as it relates to the implantation process. It is suggested that carbohydrate bearing molecules at embryonic and uterine cell surfaces play diverse roles during the implantation process that are

similar to the roles suggested for these molecules in other biological contexts.

2. INTRODUCTION

As a general principle, cell surfaces and extracellular matrices are constituted of proteins and lipids that are often variously substituted with oligosaccharides. Due to their large hydration spheres, charge characteristics and extended structures, oligosaccharides are highly exposed and participate in interactions that take place at these extracellular sites. In some cases, glycosylation may simply serve to protect polypeptides from proteolytic degradation (1). In other cases, glycosylation may serve a

Table 1. Representative glycoconjugate structures*



* Representative structures are shown. Structural microheterogeneity occurs in almost all classes. Conserved core structures are indicated by dashed boxes. Arrows indicate reducing termini of oligosaccharides. For simplicity, linkages and anomeric configurations have been omitted. Symbols: □, N-acetyl-D-glucosamine; △, N-acetyl-D-galactosamine; ◇, D-mannose; Δ, L-fucose; ? D-xylose; Λ, sialic acid; ▽, D-glucuronic acid; ●, glucose; S, sulphate

much more dynamic role including modulation of cell adhesive interactions, growth factor binding and signaling, regulation of enzyme activity and regulation of protein phosphorylation states (2-4). The type of oligosaccharide or subtleties of oligosaccharide structure are key determinants of these functions. Thus, factors that modulate the ability of a cell to express certain oligosaccharide structures, i.e., glycosyltransferases and glycosidases, as well as to produce the proteins and lipids that carry these carbohydrates control the glycobiology of cells and tissues. While core glycosylation of proteins is generally efficient, modification of terminal structures, polymerization and epimerization steps often are not.

These factors give rise not only to oligosaccharide heterogeneity within given glycoprotein classes, but also oligosaccharide microheterogeneity at specific glycosylation sites within a single protein species (5). Table 1 presents a summary of typical oligosaccharides associated with proteins and lipids discussed in this review. It is emphasized that these are generalized structures and that microheterogeneity gives rise to many variants. Table 2 summarizes functions associated with these different glycoconjugate classes. The reader is referred to more general reviews on glycoproteins, proteoglycans and glycolipids for more details on the structure and biology of these molecules (6-8). In this review, we will consider the

Table 2. Glycoconjugates and Associated Functions¹

Glycoconjugate Type	Functions
<i>N-Linked glycoproteins</i>	<u>Diverse</u> ; including promotion of cell attachment; protection against proteolysis (enhancement of protein stability); intracellular protein sorting; receptor recognition and signal transduction
<i>O-linked glycoproteins (mucins)</i>	Antiadhesive and adhesive functions; protection against proteolysis; lubrication and hydration of cell surfaces; binding sites for viruses and microbes
<i>Proteoglycans/Glycosaminoglycans</i>	
• Hyaluronic acid	Hydration of intercellular spaces; mechanical protection; promotion of cell attachment.
• Keratan sulfate	Antiadhesive and adhesive functions.
• Chondroitin sulfate/dermatan sulfate	Antiadhesive and adhesive functions; growth factor/cytokine binding (dermatan sulfate and over sulfated forms of chondroitin sulfate).
• Heparin/heparan sulfate	Promotion of cell adhesion; growth factor/cytokine binding; anticoagulant; organization of extracellular matrix; modulation of cell proliferation; binding sites for viruses and microbes.
<i>Glycolipids</i>	Promotion of cell adhesion; signal transduction (as components of lipid rafts)

¹For references see text.

roles of glycoproteins, proteoglycans and glycolipids as they pertain to the embryo implantation. Evidence will be presented indicating that these molecules play multiple, diverse roles in this critical biological process.

3. N-LINKED GLYCOPROTEINS

N-linked glycoproteins are defined as those proteins containing one or more oligosaccharides covalently attached via N-acetylglucosamine to asparaginyl groups. A distinguishing feature of N-linked glycoprotein assembly is the *en bloc* transfer of a large oligosaccharide attached to a lipid anchor, dolichylpyrophosphate, to nascent polypeptide chains. This reaction is inhibited efficiently by the antibiotic, tunicamycin (9). Treatment of mouse embryos with tunicamycin inhibits embryo attachment and outgrowth *in vitro* (10). Mouse embryos harboring a null mutation in the first enzyme involved in dolichol-linked oligosaccharide assembly, ALG7, die shortly after implantation (11). Thus, to some extent, the effects of tunicamycin on embryo attachment activities appears to reflect side effects of the drug. No particular embryonic N-linked structures have been implicated in embryo attachment; however, many cell surface and extracellular matrix proteins implicated in embryo implantation contain N-linked oligosaccharides. Thus, the requirement for N-glycosylation may reflect a general role for these modifications in protein transport or stability (1). Following transfer to protein cores, N-linked oligosaccharides are further modified by the action of multiple glycosidases and glycosyltransferases (6). Mouse null mutations in a number of the genes encoding such enzymes have been created; however, none are reported to have an implantation phenotype (11-13). Therefore, while the tunicamycin experiments indicate that N-linked oligosaccharide addition to embryonic proteins are required to support embryo attachment and outgrowth, the precise nature of these structures does not appear to be critical.

With regard to the uterus, considerably more is known about the control of N-linked glycoprotein assembly. Steroid hormones play powerful roles in this regard. Studies in rodent models have demonstrated that estrogen markedly stimulates various activities associated with N-linked

glycoprotein synthesis. In contrast, progesterone, while having little effect on its own, antagonizes estrogen action, in this regard (14-17). The molecular basis underlying these effects is not known. No genes encoding glycosyltransferases have been shown to be regulated directly by steroid hormone receptors. Thus, the general activation of glycoprotein assembly systems observed following estrogen treatment in rodents appears to be downstream of the direct actions of estrogen receptors. The availability of estrogen receptor- α and - β null mice (18,19) provide an opportunity to determine where these receptors act in the overall process leading to activation of uterine glycoprotein assembly; however, detailed studies in cell culture models identifying the events leading to increased N-linked glycosyltransferase expression following steroid hormone treatment are equally warranted. Strong effects of ovarian steroids on uterine glycoprotein assembly systems are observed in other species as well (20-24).

N-linked lactosaminoglycans are expressed by mouse uterine epithelial cells, although the identity of the protein carriers has not been determined (16,25-27). As noted above, estrogen strongly stimulates expression of lactosaminoglycan-bearing glycoproteins while progesterone antagonizes this response. Lactosaminoglycans have been implicated as partners in cell adhesion processes involving cell surface galactosyltransferase (28) and, as discussed below, certain carbohydrates implicated in implantation-related events have lactosaminoglycan backbone structures, i.e., LNF-I and Le(y) (29-31). Treatment of mouse uterine epithelia *in vitro* with agents that interfere with galactosyltransferase activity compromises uterine epithelial cell adhesion processes (32). Thus, it is possible that lactosaminoglycans help maintain tight associations among uterine epithelial cells and/or underlying basal lamina.

4. MUCINS

Mucins are defined as proteins multiply substituted with oligosaccharides in O-linkage to serine or threonine residues via N-acetylgalactosamine. In contrast to N-glycosylation, mucin oligosaccharides are built upon

proteins cores post-translationally via the sequential addition of sugars provided by sugar nucleotide donors by a series of glycosyltransferases in the smooth endoplasmic reticulum and Golgi apparatus (6). Mucin oligosaccharides can range in size from 2 to 15 sugars and are often negatively charged due to the presence of sialic acids (6). Many proteins that contain a few O-linked oligosaccharides are not generally thought of as mucins, e.g., LDL receptor (33). Rather, the term is usually applied to proteins that carry many, e.g., 20 or more of these oligosaccharides clustered along the protein core. The number and density of these substitutions result in protein regions that are inaccessible to external reagents. These features make the protein core highly resistant to enzymatic attack or unavailable to react with antibodies directed at protein core determinants (33). Underglycosylation of mucin core proteins occurs in many tumors exposing neoepitopes that have been used as diagnostic tools for cancer progression (34). Mucin glycoproteins fall into two general classes: 1) transmembrane and 2) secreted or soluble. β -D-Galactosaminides have been used to inhibit mucin oligosaccharide assembly by competing for the enzymes and substrates used to extend the oligosaccharide beyond the linkage sugar (35). Mammalian mucin genes (MUCs) from MUC1 to MUC18 have been identified (36-40). Of these, only a few (MUC1, MUC4, MUC6 and MUC8) have been reported in the uterus. Mucins also coat the surfaces of horse embryos and may play a similar antiadhesive role as proposed in the uterus (41,42).

It has long been recognized that the uterine surface is covered with a dense glycoprotein coat that thins nearing the time of implantation in most species (43). More recently, it has been shown that mucin glycoproteins are major components of this coating (44). The mucin core proteins that are expressed by uterine epithelium are the two transmembrane mucins, MUC1 and MUC4, and the secreted mucins, MUC6 and MUC8. Of these, MUC1 has been the most intensely studied although there are many similarities and interesting biological activities associated with MUC4 that warrant further investigation in the context of implantation physiology (45).

MUC1 has a large, heavily glycosylated extracellular domain and is highly expressed at the apical surface of uterine luminal and glandular epithelia in all species examined to date (46-54). During conversion to a receptive state, MUC1 is lost either throughout the luminal epithelium (49,51-54) or locally at the implantation site (48). In the former case, this is due to the actions of ovarian steroids. In the latter case, this is presumably due to factors produced by the embryos, although none have been yet identified. These observations suggest that loss of MUC1 is required for generation of a receptive uterine state. Consistent with this, various *in vitro* studies demonstrate that MUC1 directly inhibits embryo attachment (55). There is evidence in humans that at least a subset of MUC1 oligosaccharides carry selectin ligands and, therefore, potentially promote adhesive interactions (56); however, there is no evidence, in any system, that selectins play a role in embryo attachment and selectin null mice display no implantation defects (57,58). Thus, the

general model is that MUC1 is a dominant inhibitor of embryo attachment, consistent with the antiadhesive role proposed for this glycoprotein in other systems (33). Other elegant studies indicate that MUC4 plays a similar role with regard to embryo attachment indicating redundancy of function (45).

While ovarian steroids profoundly regulate uterine MUC1 expression in mice, no direct regulation of the MUC1 gene by liganded or unliganded steroid hormone receptors has been demonstrated in spite of careful examination on this point (59). These observations suggest that other downstream factors directly regulate MUC1 gene expression in response to steroid hormones. Recent studies indicate that several proinflammatory cytokines markedly stimulate MUC1 expression. Collectively, these studies indicate that both STAT1 (60) and NF κ B/Rel family transcription factors (E. Lagow and D.D. Carson, submitted) contribute to cytokine responsiveness of the MUC1 gene. Studies of cytokine responsiveness of tumor cell lines indicate that while activity of the 1.4 kb 5' flanking region of the MUC1 promoter is greatly (5-20-fold) stimulated by cytokines, only modest (<2-fold) changes in MUC1 protein or mRNA expression are observed under the same conditions. In contrast, studies of primary cell cultures indicate that MUC1 expression is quite low unless stimulated by cytokines (E. Lagow; J. Julian, J. O'Connor and D. D. Carson, unpublished studies). Therefore, it seems that while key aspects of MUC1 gene regulation can be revealed by studies of tumor cells, other features of normal regulation may be obscured. In addition, there is evidence in mammary tumor cells that cytokine-mediated translational control over MUC4 expression occurs (45). In any event, it is likely that cytokines are important physiological regulators of MUC1 expression and good candidates for downstream mediators of steroid hormone actions on MUC1 expression in the uterus. Conversely, factors that antagonize cytokine actions, e.g., SOCS proteins (61), are candidate suppressors of MUC1 expression.

In some species, e.g., rabbits (48) and humans (27), MUC1 is highly expressed during the receptive phase in most uterine epithelia; however, it is lost at sites of embryo attachment (48). These results indicate that localized MUC1 removal can occur and that embryos of these species can trigger this response. The embryo-derived factors responsible for activated removal or "shedding" of MUC1 are not known although a number of candidate molecules, including hCG, have been tested (A. Thathiah and D.D. Carson, unpublished studies). Surprisingly little is known about the mechanism of MUC1 shedding in any system although it is a well-recognized process (62-64). The shedding event separates the large, extracellular domain from the cytoplasmic tail (63). This may involve the action of a cell surface protease or possible an activity that causes dissociation of the non-covalent complex between these domains. In this regard, MUC1 and MUC4 are synthesized as a single polypeptide that is proteolytically cleaved intracellularly, but remains tightly associated during intracellular transit and at the cell surface. Thus, activities that could cause dissociation of

this complex without proteolysis, e.g., protein kinases, also might play a role in mucin shedding.

5. PROTEOGLYCANS

Proteoglycans are defined as proteins bearing one or more polysaccharides called glycosaminoglycans. In general, glycosaminoglycans consist of large, linear, highly negatively charged polysaccharides composed of repeating disaccharides of uronic acid and hexosamine. Each disaccharide unit usually carries 1-2 sulfate residues. Almost all of the glycosaminoglycan polymerization and carbohydrate chain modification reactions occur in the Golgi apparatus (7). Hyaluronic acid is the simplest form consisting of a disaccharide repeat (N-acetylglucosamine, glucuronic acid) and not attached to protein. Nonetheless, a single hyaluronate molecule may be millions of daltons in size. The chondroitin sulfate family is assembled on protein cores and contains repeating disaccharides (N-acetylgalactosamine and glucuronic or iduronic acid) usually carrying one sulfate per disaccharide and ranging in size from 20 – 50,000 daltons. Keratan sulfate is actually a sulfated form of lactosaminoglycan and deviates from the general description of glycosaminoglycans in that it does not contain uronic acids and can be branched. The disaccharide units are variously sulfated and consist of N-acetylglucosamine and galactose. Moreover, keratan sulfate can occur on N-linked or O-linked core structures linked to protein or glycolipids (6,7). The classification of keratan sulfate as a glycosaminoglycan reflects a historical designation based on the chromatographic behavior of these polysaccharides rather than structural features. The heparan sulfate family is the most complex glycosaminoglycan class since the disaccharide units can vary considerably depending on whether the glucosamine residues are N-acetylated, N- or O-sulfated and whether glucuronic or iduronic acids are present and sulfated. As a consequence, heparan sulfates have distinct sequence motifs within them that may be recognized more or less specifically by various proteins (65). This is currently an intensive field of study with regard to extracellular matrix and growth factor interactions with heparan sulfate. Recent work indicates that sequence variation also occurs in chondroitin sulfate polysaccharides although not to the same extent as heparan sulfates (66).

Early studies in mice revealed that heparan sulfate synthesis increased 4-5 fold at the peri-implantation stage and was required for embryo attachment and outgrowth *in vitro* (67,68). Heparan sulfate proteoglycans also participate in adhesive interactions occurring between human trophoblastic and uterine epithelial cell lines, a model used to mimic aspects of human implantation (69,70). The heparan sulfate proteoglycans, syndecan (71) and perlecan (69), are expressed by mouse embryos at the peri-implantation stage, although syndecan appears to be disposed toward the inner surface (blastocoel cavity) of trophectoderm while perlecan is found on the external surface, i.e., where it might interact with uterine cells and ECM components. These latter observations led to the suggestion that perlecan participates in early stages of the implantation process. In this regard, studies with a delayed

implanting mouse model demonstrated that perlecan expression also was delayed and restored upon estrogen activation (72).

As mentioned above, one suggestion for the roles for these proteoglycans is as adhesion-promoting molecules. Another role is potentiation of growth factor activities. Heparan sulfate binds a wide variety of growth factors and cytokines (7,65). In some cases, particular heparan sulfate structures appear to be recognized by these proteins (65). In addition to binding growth factors, heparan sulfate binding is also observed for several growth factor receptors, e.g., FGF receptors (73) and c-met (74), and is required for signal transduction. HB-EGF is induced locally at implantation sites in mice and binding to mouse embryos requires both the erbB-binding and heparan sulfate-binding motifs of the growth factor (75).

In spite of these provocative correlative studies, genetic tests have not confirmed the requirement for heparan sulfate proteoglycans in initial stages of the implantation process. Syndecan, glypican and perlecan null mice have been created without obvious implantation phenotypes (76). Moreover, knockouts of genes encoding a number of enzymes involved in heparan sulfate biosynthesis also have been created, again with no reported impairment of implantation (76). It is possible that heparan sulfate proteoglycans play a more subtle, supportive role in the implantation process, e.g., growth factor binding and modulation of aspects of embryonic cell growth. Functional gene redundancy also might account for the lack of effects observed in these null models. Such redundancy can occur both for enzymes involved in heparan sulfate assembly (76) and for proteoglycan core proteins (77). The creation of double nulls could address this issue. Perlecan is a good candidate to test since there appears to be only one gene (76); however, in this case a severe post-implantation phenotype is observed involving many tissues and only a few embryos survive after birth and none to sexual maturity. It will be necessary to use tissue-specific or inducible knockout or knockdown approaches to circumvent this problem.

6. GLYCOLIPIDS

The vast majority of studies on uterine and embryonic glycoconjugates have focused on glycoproteins and proteoglycans. The reasons for this no doubt include the availability of probes that can detect not only the carbohydrate structures, but also expression of the protein cores by immunohistochemistry and *in situ* hybridization. Nonetheless, it should be noted that many of the antibody and lectin probes used to detect carbohydrate structures detect these structures on glycolipids as well as glycoproteins. A notable example is the lactosaminoglycan family (78). Expression of most neutral and globo- series glycosphingolipids do not change during the cycle in humans, mice and rabbits; however, expression of certain charged species substantially increase during the receptive phase (79-81). In addition, activities of enzymes involved in assembly or degradation of these charged glycolipids change similarly to account for this response (82). Other

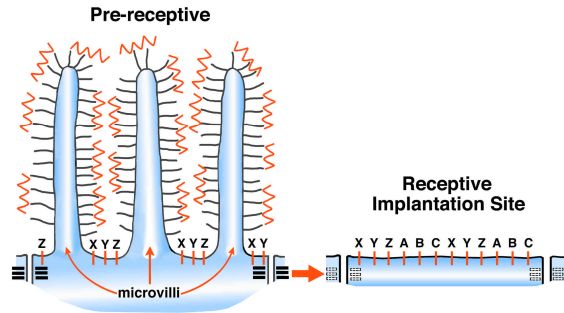


Figure 1. Conversion of the uterine epithelium to a receptive state. During the prereceptive state, the apical surface of the uterine epithelium schematically shown expresses large microvilli with an abundant complement of antiadhesive transmembrane (black lines extending from microvilli) and secreted (red zig-zag lines above microvilli) mucins. Various adhesion promoting molecules, including glycoconjugates, may be expressed, but are functionally inaccessible (-X, -Y, -Z). Junctional complexes (°) are present. Both apical microvilli and mucins are lost during transition to a receptive uterine state, although in some species this may only occur at the implantation site. Additional adhesion-promoting molecules (-A, -B, -C) may appear during the receptive sphase. Junctional complexes also are compromised.

studies indicate that expression of certain lactosaminoglycan-related structures, i.e., LNF-I (30), CD15 (83) and Le(y) (31), also increase markedly during the receptive phase in various species. It should be noted that in studies in which changes have been monitored by immunostaining it is not clear to what extent these changes reflect alterations in expression of glycoproteins or glycolipids. It also is of concern that the organic solvents frequently used to fix tissues for immunostaining may at least partially extract glycolipids from tissues. Another potentially confounding factor is the observation that neuraminidase pretreatment can expose additional cryptic sites for antibody binding, presumably by removing sialic acid residues on these structures that inhibit antibody binding (84). Thus, observed changes in expression of a given carbohydrate structure might reflect alterations in sialyltransferase, endogenous neuraminidase or various other glycosyltransferase activities involved in assembly of these structures. Careful studies examining the spatial distribution and biochemical levels of these glycoconjugates are required to make definitive statements. Nonetheless, genetic ablation of a number of key enzymes in glycolipid assembly have been made (13). While in some cases this results in embryonic lethality, in no case has an implantation defect been reported.

Functionally, both LNF-I and Le(y) have been implicated in mediating aspects of embryo-uterine interactions in mice (29,31). Mouse embryos bind LNF-I structures although the receptors for these structures have not been identified. Le(y) is found on both mouse embryo and uterine epithelial cells surfaces where they potentially can support attachment via homotypic interactions. An inhibitor of glycosphingolipid synthesis, N-

butyldeoxygalactonojirimycin, does not affect post-implantation development in the mouse (85); however, studies of preimplantation development have not been performed to determine if these glycolipids are likely to play a role in either blastocyst development or embryo-uterine interactions. While it remains uncertain whether glycolipids play an important role in early stages of the implantation process, enough provocative data exists to warrant further examination.

7. SUMMARY AND FUTURE DIRECTIONS

Expression of a variety of glycoconjugates change markedly as the embryo develops to an implantation competent state and the uterus matures to a receptive state. A number of *in vitro* and *in vivo* studies have tested the potential functionality of these molecules in the implantation process. The most definitive of these *in vivo* functional tests is genetic ablation of genes encoding core proteins bearing oligosaccharides or key enzymes involved in glycoconjugate assembly. No gene "knockout" has revealed an essential role for an individual glycoconjugate in early stages of the implantation process. Consequently, one interpretation is that glycoconjugates do not play critical roles in this event. Another interpretation is that functional redundancy exists for glycoconjugates involved in this vital biological process. Roberts *et al.* (86) have shown that mutation rates of placental genes exceed these of other genes. One interpretation of these observations is that the fetal maternal interface is a genetic "battleground" in which each side is constantly looking for a new advantage. In the case of trophoblast, the embryo must establish contact with the maternal system to provide nutrients and a suitable environment for its growth while protecting itself from the maternal immune system. In the case of maternal tissue, the uterus must find ways to support embryonic and placental growth while protecting itself from excessive invasion, immune reactions to the embryo and, in many species, maintaining hemostasis as trophoblast physically invade uterine arteries. Different species have developed significantly different approaches to establishing fetal-maternal contact, perhaps reflecting the genetic struggle to maintain the transient alliance these tissues. In fact, no knockouts of genes encoding extracellular matrix components or cell adhesion molecules have revealed an implantation phenotype. Thus, there may be no single entity one could call an "embryo receptor". Rather, the uterine epithelial surface probably contains many constituents, including various glycoconjugates, that can perform this role. This suggestion is consistent with *in vitro* observations demonstrating considerable plasticity of blastocysts with regard to attachment and outgrowth activities, including upon biological substrates that are not encountered during the implantation process, e.g., collagen type II (87).

If this suggestion is true, the dominant factor controlling the implantation process may be preventing it (Figure 1). Multiple molecules capable of supporting embryo adhesion may co-exist with mucins in a pre-receptive state. Others may arise as the uterus transitions to a receptive state, e.g., $\alpha_v\beta_3$ integrins in humans.

Nonetheless, the abundance, highly extended structure and antiadhesive nature of mucins compared to adhesion promoting molecules provides a massive barrier to embryo attachment. The presence of apical microvilli at the pre-receptive state further impairs intimate access to the epithelial cell surface. Microvilli may be lost either via retraction or the generation of "pinopodes" (88). Loss of microvilli (43,89) as well as mucins during transition to the receptive state creates access to a spectrum of adhesion promoting molecules, including various glycoconjugates. *In vitro* studies demonstrate that mucins alone can inhibit embryo or cell attachment, regardless of the cell surface adhesion systems present and are, therefore, dominant factors. Interestingly, recent evidence indicates that pinopodes are devoid of MUC1 consistent with the notion that these structures may facilitate embryo-uterine interaction (90). Rigorous *in vivo* tests of this hypothesis must be done with regard to implantation and appropriate approaches are now available to do so. Moreover, it will be important to determine what factors control mucin expression and removal to understand how different species create access to the uterine epithelial cell surface to initiate implantation.

8. ACKNOWLEDGEMENTS

I am extremely grateful to Dr. C. Farach-Carson, Dr. C. Kirn Safran, T. Ahrens, M. Brayman, E. Lagow and A. Thathiah for their critical reading of this manuscript and their many helpful discussions. I appreciate the excellent secretarial assistance of Ms. Sharron Kingston and the graphics work of Mrs. Margie Barrett. Relevant work in the author's lab is supported by NIH grants HD 25235 and HD 29963 (to D.D.C.).

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Key Words: Glycobiology, Implantation, Mucins, Proteoglycans, Uterus, Review

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