AN ABSTRACT OF THE THESIS OF

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Title: Steroids Regulate α2,6-Sialic Acid-Containing Glycoconjugates in Murine Uterine Epithelium at the Time of Implantation

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Sialic acids are involved in many cellular interactions. They can serve as an adhesion ligand or act as an inhibitor to cellular adhesion by charge repulsion or by masking potential ligands. Although sialic acids are implicated in the process of blastocyst implantation, their expression and regulation in uterine epithelium of mice have not been studied. The lectin, *Sambucus nigra* (SNA) specifically recognizes α2,6-linked sialic acids, which are involved in cell recognition phenomena. It was used to probe frozen uterine sections from mice during days one through six of pregnancy. SNA staining was most intense at the apical surface of uterine epithelial cells on day one of pregnancy, decreased gradually through day four, and was undetectable by day five.

The role of the steroid hormones, estrogen and progesterone, in regulating the expression of α2,6-linked sialic acids was studied in uterine sections from mice during the estrous cycle and in ovariectomized mice given hormone replacement
using SNA. SNA staining of these sections during the estrous cycle showed that the expression of α2,6-linked sialic acids was stage dependent. Staining was most intense in uterine sections from mice in estrus, and was not detected in sections from mice in diestrus. In ovariectomized mice, staining was most intense in mice injected with estradiol alone, and no staining was evident in mice injected with progesterone alone. These results suggest that the expression of α2,6-linked sialic acids decreases during the time of implantation and that estrogen stimulates and progesterone inhibits its expression.

β-Galactoside α2,6-Sialyltransferase (α2,6-ST) is the enzyme that links sialic acids to Galβ1-4GlcNAc termini of N-linked oligosaccharides. In order to investigate the mechanism behind the hormonal regulation of α2,6-linked sialic acids, the expression of α2,6-ST was followed in uterine sections from mice during early pregnancy, during the estrous cycle, and in ovariectomized mice given hormone replacement. In-situ hybridization was performed using digoxigenin labeled RNA probes to characterize α2,6-ST mRNA levels in uterine sections. Expression of α2,6-ST protein was also measured in uterine sections with a polyclonal antibody against α2,6-ST. The expression of α2,6-ST mRNA and protein correlated well with the timing of the appearance of α2,6-linked sialic acids.

These results show that the expression of α2,6-linked sialic acids on the surface of mouse uterine epithelium decreases at the time of implantation and
furthermore, that this decrease is due to the regulation of α2,6-ST by the steroid hormones. α2,6-linked sialic acids may serve to inhibit cellular adhesion by creating a charge repulsion, or by masking potential binding sites. Removal of this inhibition may permit blastocyst implantation.
Steroids Regulate α2,6-Sialic Acid-Containing Glycoconjugates in Murine Uterine Epithelium at the Time of Implantation

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Georgeen S. Gaa-Bulseco, Author
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# TABLE OF CONTENTS

## CHAPTER 1

1. **Introduction**
   - Overview
   - The Implantation Reaction
   - Steroid Hormones: Estrogen and Progesterone
   - Levels during the Estrous Cycle
   - Levels of Estrogen and Progesterone During Early Pregnancy
   - Role of Estrogen and Progesterone during Implantation
   - Changes in the Uterine Epithelium during Implantation
     - Morphological Changes
     - Surface Charge
     - Uterine Epithelial Surface Glycoconjugates
     - Sialic Acids
     - Glycosyltransferases
     - Summary

## CHAPTER 2

39. **Materials and Methods**
   - Materials
   - Animals
   - Isolation of uterine epithelial cells
   - Extraction of Uterine Epithelial Fragments
   - SDS-PAGE and Lectin Transblots
   - Lectin Histochemistry
TABLE OF CONTENTS, CONTINUED

Immunohistochemistry 46
In situ Hybridization 47
Preparation of RNA Probe 47
Preparation and Pretreatment of Tissue Sections 48
Prehybridization, Hybridization and Posthybridization 50
Immunological Detection 50

CHAPTER 3 52
Results 52
Levels of α2,6-linked sialic acid in uterine epithelial cell extracts 52
Changes in extracts from mice in early pregnancy 54
Changes in extracts from mice during the estrous cycle 56
Expression of α2,6-linked sialic acids in uterine sections 56
In Early Pregnancy 59
During the Estrous Cycle 59
In Ovariectomized Mice Given Hormone Replacement 63
Expression of α2,6- Sialyltransferase mRNA in Uterine Epithelial Cells 66
In Early Pregnancy 66
During the Estrous Cycle 69
In Ovariectomized Mice Given Hormone Replacement 69
Expression of α2,6-Sialyltransferase protein in uterine epithelium 72
During early pregnancy 72
During the estrous cycle 73
In ovariectomized mice given hormone replacement 73

CHAPTER 4 77
Discussion 77
TABLE OF CONTENTS, CONTINUED

Decrease in Glycoconjugates containing \( \alpha2,6 \)-linked sialic acids at the time of implantation 77

Factors Involved in the Regulation of the Levels of \( \alpha2,6 \)-linked sialic acids 81
  Decrease in the levels of \( \alpha2,6 \)-sialyltransferase 81
  Other Factors 83

Role of Estrogen and Progesterone 85

Role for a Decrease in Levels of \( \alpha2,6 \)-Sialoglycans During Implantation 89

Conclusion 90

REFERENCES CITED 95
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cross section of a mouse uterus.</td>
<td>7</td>
</tr>
<tr>
<td>2.</td>
<td>Levels of estrogen and progesterone during the estrous cycle and early pregnancy in laboratory mice.</td>
<td>11</td>
</tr>
<tr>
<td>3.</td>
<td>Structure of sialic acid.</td>
<td>27</td>
</tr>
<tr>
<td>4.</td>
<td>The $\alpha_2,6$-sialyltransferase sialylmotif domain.</td>
<td>35</td>
</tr>
<tr>
<td>5.</td>
<td>Appearance of vaginal smears showing the stages of the estrous cycle.</td>
<td>42</td>
</tr>
<tr>
<td>6.</td>
<td>Diagram of pBluescript II K/S+ with $\alpha_2,6$-sialyltransferase inserted at the EcoRI site in the open reading frame.</td>
<td>49</td>
</tr>
<tr>
<td>7.</td>
<td>Isolated uterine epithelial flakes.</td>
<td>53</td>
</tr>
<tr>
<td>8.</td>
<td>Changes in $\alpha_2,6$-linked sialic acids in extracts from mice during early pregnancy.</td>
<td>55</td>
</tr>
<tr>
<td>9.</td>
<td>Changes in $\alpha_2,6$-linked sialic acids in uterine epithelial cell extracts from mice in the different stages of the estrous cycle.</td>
<td>58</td>
</tr>
<tr>
<td>10.</td>
<td>$\alpha_2,6$-linked sialic acid in frozen sections of mouse uterus during early pregnancy.</td>
<td>60</td>
</tr>
<tr>
<td>11.</td>
<td>Implantation site probed with SNA.</td>
<td>61</td>
</tr>
<tr>
<td>12.</td>
<td>Frozen sections of mouse uteri during early pregnancy treated with neuraminidase prior to probing with SNA.</td>
<td>62</td>
</tr>
<tr>
<td>13.</td>
<td>$\alpha_2,6$-linked sialic acid in frozen sections of mouse uterus during the various stages of the estrous cycle.</td>
<td>64</td>
</tr>
<tr>
<td>14.</td>
<td>$\alpha_2,6$-linked sialic acid in sections of uteri from ovariectomized mice.</td>
<td>65</td>
</tr>
<tr>
<td>15.</td>
<td>Expression levels of $\alpha_2,6$-ST mRNA in uterine epithelial cells from mice in early pregnancy.</td>
<td>67</td>
</tr>
<tr>
<td>16.</td>
<td>In situ hybridization control sections of mouse uteri during early pregnancy</td>
<td>68</td>
</tr>
<tr>
<td>17.</td>
<td>Expression levels of $\alpha_2,6$-ST mRNA in uterine epithelial cells from mice during the various stages of the estrous cycle.</td>
<td>70</td>
</tr>
<tr>
<td>18.</td>
<td>Expression of $\alpha_2,6$-ST mRNA in uterine epithelial cells from ovariectomized mice given hormone replacement.</td>
<td>71</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>19.</td>
<td>Expression of α2,6-ST protein in uterine epithelial cells from mice during early pregnancy.</td>
<td>73</td>
</tr>
<tr>
<td>20.</td>
<td>Expression of α2,6-ST protein in uterine epithelial cells from mice during the estrous cycle.</td>
<td>75</td>
</tr>
<tr>
<td>21.</td>
<td>Expression of α2,6-ST protein in uterine epithelial cells from ovariectomized mice given hormone replacement.</td>
<td>76</td>
</tr>
<tr>
<td>22.</td>
<td>Comparison of expression levels of α2,6-ST mRNA, protein and α2,6-linked sialic acid.</td>
<td>88</td>
</tr>
<tr>
<td>23.</td>
<td>Schematic diagram of a possible model for the initial stage of implantation.</td>
<td>92</td>
</tr>
</tbody>
</table>
Embryo implantation is a process by which the blastocyst becomes attached to the maternal uterine wall and it is regulated by the hormones estrogen and progesterone (Psychoyos, 1986). In rodents, progesterone converts the uterus to a sensitized state, which becomes receptive to blastocyst attachment following a transitory increase in estrogen on day four of pregnancy (Huet-Hudson and Dey, 1990; McCormack and Greenwald, 1974). Implantation is initiated when contact is made between the apical membrane of the trophoblast cells of the blastocyst and the apical membrane of the luminal epithelium of the uterus.

The apical surface of uterine luminal epithelial cells is usually non-adhesive, which is a characteristic common to all epithelial cells that line tissues. However, during the receptive period for embryo implantation, the apical surface of uterine epithelial cells becomes transiently adhesive, which allows attachment of the blastocyst. This change could result from redistribution or expression of new
adhesion molecules, or the loss of inhibitor molecules that block adhesion. Some of the changes that have been reported to occur include a reduction in the thickness of the glycocalyx (sugar coat) and a decrease in negative surface charge (Anderson and Hoffman, 1984; Hewitt et al., 1979; Morris and Potter, 1984). Changes in the composition of glycoconjugates have also been observed with the use of lectins and antibodies (Braga and Gendler, 1993; Kimber, 1994; Surveyor et al., 1995).

Recently, there has been increased interest in sialic acids and their role in cellular interactions. Sialic acids occur primarily as terminal sugars with different linkages to a variety of oligosaccaride glycans. They are involved in many cell adhesion processes in which adhesion proteins recognize specific sialylated structures. For example, members of the selectin family, which are involved in the adhesion of leukocytes to endothelial cells at sites of inflammation, recognize and bind to sialylated structures such as sia1y1-Lewis^x (Berg et al., 1991). Sialic acids can also have a negative effect on adhesion. Due to their terminal location and negative charge, they can inhibit cellular interactions through steric hindrance and negative repulsion (Varki, 1997). Although a decrease in neuraminidase-sensitive surface charge at the time of implantation has implied an overall decrease in sialic acids (Hewitt et al., 1979), direct studies are lacking, especially on the expression of glycoconjugates containing a specific type of sialic acid linkage.

The goal of this study was to directly examine the steroid mediated changes in (1) the expression of α2,6-linked sialic acids and (2) changes in the expression of
a regulatory enzyme in the biosynthetic pathway of α2,6-linked sialic acids.

Expression of this sugar in tissue extracts and on tissue sections was accomplished with the use of a lectin, **Sambucus nigra** agglutinin that specifically recognizes α2,6-linked sialic acids (Shibuya et al., 1987). Detailed study of a specific linkage allows one to detect subtle changes in expression levels, which may be obscured if probing for total sialic acids. The reason for studying specifically the α2,6-linked sialic acids is its role in cell recognition and adhesion in other cellular systems. *H. influenzae* viruses isolated from human bind to the NeuAca2,6-Gal sequence (Rogers and Paulson, 1983). The lymphocyte B-cell surface receptor CD223 binds specifically to oligosaccharides containing α2,6-linked sialic acids (Keim et al., 1994). A positive correlation has also been made between the metastatic potential of murine colon cancer cell lines and α2,6-linked sialoglycoconjugate expression (Bresalier et al., 1990).

This study also examined whether there is any correlation between the levels of α2,6-linked sialic acids and the expression of the enzyme that synthesizes this linkage. **3-Galactoside α2,6-Sialyltransferase (α2,6-ST)** is the enzyme that mediates the transfer of sialic acid to exposed Gal l-4GlcNAc termini of N-linked oligosaccharides. Following the expression of this enzyme has provided insights into the mechanism involved in the expression of glycoconjugates containing α2,6-linked sialic acids. The availability of cDNA and a polyclonal antibody against α2,6-
ST made this study feasible. This study also examined whether the steroid hormones estrogen and progesterone are involved in the expression of α2,6-linked sialic acids.

Information gained from this study provides insights into changes occurring at the apical surface of uterine epithelial cells and it may provide a better understanding of how sialic acids are involved in promoting a receptive uterus. Following the expression of α2,6-ST also provides insights into the mechanism involved in the expression of glycoconjugates containing α2,6-linked sialic acids.

**THE IMPLANTATION REACTION**

Implantation involves a series of events that lead to an intimate union between the blastocyst and the uterine epithelium. Implantation events have been categorized into the apposition stage, the adhesion stage, and in those species where it occurs, the invasive stage. This union allows the exchange of nutrients and waste products between maternal and fetal tissues, and is vital for the survival of the offspring. Prior to implantation, the uterus is non-receptive to blastocyst attachment. As implantation nears, changes take place that transforms the uterus into a receptive state. Receptivity lasts for a short period of time before the uterus returns to a non-receptive, or refractory state. It is regulated in mice by the steroid hormones estrogen and progesterone (Psychoyos, 1986). Synchronized development of the activated embryo to the blastocyst stage and differentiation of the uterus to the receptive state are crucial to the success of implantation.
The mammalian embryo travels through the reproductive tract after fertilization where it continues to divide and develop into a blastocyst. The blastocyst consists of two cell types. The outer layer of cells are the trophoblasts, which surround the cavity of the blastocyst and will form part of the placenta. The second cell type, the cells of the inner cell mass (ICM) is concentrated at one end of the blastocyst and will form the embryo proper. A non-adhesive zona pellucida surrounds the entire blastocyst and may contribute to preventing premature attachment of the blastocyst as it moves toward the uterus (Dickmann and Noyes, 1961). The major goal of the blastocyst at this time is to reach the uterus where it will implant to obtain nourishment.

The uterus is composed of the endometrium and myometrium. The endometrium consists of luminal and glandular epithelial cells, stromal cells, and vasculature, and the myometrium is made up of smooth muscle cell layers (Fig. 1). The epithelium lining the lumen of the uterus is non-adhesive and like epithelia of other mucosal tissues, provides a barrier to bacterial infection. As the blastocyst approaches the uterus, it hatches from its surrounding zona pellucida and develops into an activated state, ready for implantation.

Implantation begins when the blastocyst comes into close contact to the uterine luminal epithelial cells (Enders and Schlafke, 1969). At the first stage of attachment, the apposition stage, contact is intimate, but the blastocyst can be displaced easily at this stage with gentle perfusion of the uterine lumen. The
adhesion stage starts when a physical attachment between the trophoblast and the luminal epithelial cells of the uterus occurs. Attempting to separate the blastocyst from the uterus at this point may cause cellular damage (Martin, 1984; Milligan and Martin, 1984).

Many changes have taken place in the uterus during these initial stages of implantation. The non-adhesive uterine epithelial cells lining the lumen become adhesive and receptive to blastocyst attachment. The stromal cells transform into large polyploid decidual cells (Enders and Schlafke, 1967; Jones et al., 1996), and glycogen and lipids accumulate in the cytoplasm of these cells. This change in the stroma is called the Decidual Cell Reaction (DCR). Other changes include a local increase in uterine vascular permeability at the sites of implantation (Das et al., 1997; Parr and Parr, 1989) and changes in glandular secretions into the lumen (Given and Enders, 1981).
Figure 1: Cross section of a mouse uterus.

Paraformaldehyde fixed cross section of a mouse uterus in proestrus stained with haematoxylin and eosin to demonstrate cellular components. Luminal epithelial cells (LE); stroma (S); myometrium (M); uterine glands (GL); and lumen (L). Bar = 100uM.
Implantation does not proceed beyond the adhesion stage in some species. For example, implantation in ruminants is quite shallow (Guillomot, 1995). The maternal vasculature develops in close apposition to the site of embryo attachment, providing access to nourishment. In other species including humans and rodents, implantation is invasive. Trophoblast cells must penetrate the epithelium and underlying stroma to establish contact with the maternal circulation. This is known as the invasive stage.

Despite species-dependent differences in blastocyst invasiveness, the initial implantation event where the apical plasma membrane of the trophoblast attaches to the apical plasma membrane of the uterine epithelium occurs in all eutherian mammals. This event has been referred to as a “cell biological paradox” because the apical surfaces of opposing epithelial cells are usually non-adhesive and do not bind to each other (Denker, 1993). The integrity of tissues and organs depend on this non-adhesive nature of epithelial cells. Epithelial cells lining the lumen of the uterus, like epithelia of other mucosa, provide a barrier to the spread of microbial infections. Moreover, uterine luminal epithelial cells must allow sperm transit prior to fertilization. Apparently, changes must alter these properties to allow access to blastocyst attachment.

Recently, it has been hypothesized that uterine epithelial cells may lose polarity during blastocyst attachment (Denker, 1993; Glasser and Mulholland, 1993), which could increase its adhesivity. More importantly, changes at the apical surface
itself may affect or modify adhesivity. One possibility is that adhesion molecules may be redistributed or new ones expressed. Alternatively, molecules that normally block adhesion are no longer expressed. This period of receptivity to implantation is transient and regulated by estrogen and progesterone (Psychoyos, 1986). At least 24 hours of progesterone followed by a pulse of estradiol is necessary to prepare the uterus for implantation in mice (Huet-Hudson and Dey, 1990).

Successful implantation requires the synchronized development of the embryo into an activated blastocyst and differentiation of the uterus into a receptive state. The role of uterine epithelium in this receptivity may be primarily due to the removal of constraints on implantation. When uterine epithelial cells are removed from the non-receptive uterus, blastocysts attach directly to the stroma, independently of regulation (Colwell, 1969). Also, activated blastocysts have also been shown to bind to extrauterine sites (Carson et al., 1990). For these reasons, the changes that take place in uterine epithelial cells during the early attachment stage of implantation are emphasized in the following sections.

**STEROID HORMONES: ESTROGEN AND PROGESTERONE**

Sex steroids are largely responsible for the timing and coordination of reproduction, and most of the reported changes that take place in the uterus during implantation are regulated by estrogen and progesterone (Huet-Hudson et al., 1990; Kimber et al., 1990; and Surveyor et al., 1995). In order to establish whether a particular change *in vivo* is under hormonal control, a correlation between the timing...
of the change and the appearance of the hormones need to be made. Therefore, it is necessary to consider the pattern of hormone secretion during the estrous cycle and during early pregnancy in the mouse.

**Levels during the Estrous Cycle**

The estrous cycle consists of four main stages; proestrus, estrus, metestrus, and diestrus. In laboratory mice, it takes four days to complete one full cycle. During proestrus, ovarian follicles undergo growth and cellular proliferation. Estrus follows proestrus, and in this stage the mature follicles rupture to release the ovum. Following ovulation, the corpus luteum is formed from the remaining granulosa and theca cells of the ruptured follicle. It is responsible for production of progesterone through the stages of metestrus and diestrus. If copulation does not take place, the corpus luteum degenerates, and the cycle is repeated. However, if fertilization occurs, the corpus luteum remains active and continues to secrete progesterone. During the estrous cycle, serum levels of estrogen in mice peak during proestrus and levels of progesterone peak during diestrus (Walmer et al., 1992) (Fig 2).
Figure 2: Fluctuation of estrogen and progesterone during the estrous cycle and early pregnancy in laboratory mice.

Schematic diagram showing the timing of estrogen and progesterone fluctuations in mice during the estrous cycle and early pregnancy (constructed after McCormack and Greenwald, 1974 and Walmer et al., 1992). Units shown are arbitrary and do not reflect the relative levels of estrogen and progesterone. Arrow indicates initiation of attachment phase of implantation. D = diestrus, P = proestrus, E = estrus, and M = metestrus.
Levels of Estrogen and Progesterone During Early Pregnancy

Radioimmunoassay procedures (McCormack and Greenwald, 1974) showed that serum progesterone concentration is low on days one and two of pregnancy and begins to rise on day three. The concentration peaks at day six and decreases significantly by day seven (Fig. 2). Estradiol concentration, however, is high on day one and declines to low levels on days two and three. A slight elevation in estradiol occurs on day four of pregnancy, which is followed by a decline (McCormack and Greenwald, 1974). Implantation occurs early on day five (Psychoyos, 1986).

Role of Estrogen and Progesterone during Implantation

Studies on delayed implantation and embryo transplants show that both estrogen and progesterone are required in mice for successful implantation to occur. In order for the uterus in mice to be receptive to blastocyst implantation, it must first be primed with progesterone for at least 24 hours to reach a pre-receptive neutral state (Huet-Hudson and Dey, 1990). A dose of estrogen after this priming period (seen at day four of pregnancy) transforms the uterus into a receptive state. If implantation does not take place within 24 hours after the appearance of estrogen, the uterus goes into a refractory state and becomes a hostile environment for the blastocyst (Psychoyos, 1986).

Progesterone has been shown to be a major factor in preparing the uterine tissue for implantation. The corpus luteum and its role in producing progesterone for the maintenance of the uterus during early pregnancy has been well documented
(Marcus and Shelesnyak, 1970), and blocking progesterone receptors with the synthetic steroid mifepristone (RU486) stops the uterine wall from thickening and disrupts attachment of the blastocyst (Couzinet et al., 1986).

The first clue that estrogen was necessary for blastocyst attachment came from early studies on delayed implantation. Natural delayed implantation occurs in lactating rats and mice when the female mates and conceives at postpartum estrus while suckling a litter. The blastocyst remains viable but quiescent within the uterus until the litter has been weaned. Studies show that implantation can be induced before weaning by the administration of a small dose of estradiol (Krehbiel, 1941). Delayed implantation can be induced in pregnant rats and mice by ovariectomy before implantation. Progesterone is administered following ovariectomy to maintain the uterus. The timing of ovariectomy is critical. If it is performed prior to the rise of estrogen that occurs on day four of pregnancy, implantation does not occur unless estradiol is administered. If it is performed after the rise of estrogen, implantation occurs at the normal time (Yoshinaga and Adams, 1966). Successful transfer of blastocysts into hormone-treated ovariectomized mice also requires a period of progesterone treatment followed by estradiol (Humphrey, 1969; Smith and Biggers, 1968).

Many of the changes seen at the cellular level in the receptive uterus have been shown to be regulated by estrogen and progesterone. This includes morphological changes (Enders and Schlafke, 1969), changes in cell surface charge
(Hewitt et al., 1979; Morris and Potter, 1984), and changes in the expression of glycoconjugates (Kimber and Lindenberg, 1990) and structural proteins (Carson et al., 1998).

**CHANGES IN THE UTERINE EPITHELIUM DURING IMPLANTATION**

**Morphological Changes**

Ultrastructural examination of uterine epithelial cells show that these cells go through morphological changes immediately before implantation. Numerous vesicles begin to appear near the apical region of the cells (Smith and Wilson, 1974). Microvilli that line luminal epithelia become significantly shorter and fewer in number (Enders and Schlafke, 1969). There is also the appearance of large cytoplasmic protrusions that extend into the uterine lumen. These protrusions vary in size and number and have been observed in many species including rat (Ljungkvist, 1972; Parr and Parr, 1974), mouse (Parr and Parr, 1977), and humans (Nilsson, 1972). These protrusions, or pinopods as termed by Enders (1973) are sometimes seen making contact with the blastocyst (Tachi et al., 1970).

Evidence suggests that the cells are actively engaged in endocytosis just prior to implantation. Enders (1973) injected ferritin into the lumen of day five pregnant rats and observed the uptake after different time intervals. After five minutes, the ferritin distribution was sparse and found in only a few pinopods. After 10 minutes, ferritin was widely distributed in vacuoles within the pinopods and in the apical
portions of cells lacking pinopods. After 60 minutes, ferritin was found predominantly in multivesicular bodies and dense bodies in the middle of the cell. These findings are confirmed by the studies performed by Parr (1974) and was later observed in mice (Parr and Parr, 1977). They suggest that initially, pinopods are formed. Lateral edges of the pinopods start to fold in forming pockets of fluid. Closure of these pockets by typical endocytotic movement form vacuoles of trapped fluid. Pinopods containing vacuoles eventually regress back into the apical cytoplasm of the cell. This explains the presence of ferritin filled vacuoles in cells lacking pinopods. With time, the endocytosed material ends up in multivesicular and dense bodies in the middle of the cell. Most of the endocytosed material is believed to be degraded in lysozomes.

Parr (1983) went on to show that the appearance of these pinopods and the process of endocytosis at the surface of uterine epithelial cells is regulated by progesterone. Ovariectomized rats treated with progesterone or estrogen followed by progesterone exhibit endocytotic activity. There is no evidence of endocytotic activity in uterine epithelial cells of untreated rats or rats treated with estrogen alone. Endocytotic activity was also shown in pregnant mice during days two, three, and four, when progesterone dominates. No activity is observed after the surge of estradiol that occurs later on day four (Parr and Parr, 1977).

Several possible explanations are given for the occurrence of endocytosis just prior to implantation. Endocytosis could be aiding the process of uterine closure by
removing fluid from the lumen (Parr, 1983). Uterine closure appears to aid in blastocyst attachment by bringing blastocyst and uterine epithelial cells closer together. Another possible role for endocytosis is the removal from the apical surface of glycoconjugates that may inhibit adhesion (Enders and Nelson, 1973).

**Surface Charge**

Several studies show that the surface charge of uterine epithelial cells is also altered. In a number of species, the surface of uterine epithelial cells appears to be negative during the non-receptive phase. This negative charge decreases as the period of implantation nears.

Uterine epithelial cells of rabbits in estrus, early pseudopregnancy and pregnancy were examined with electron microscopy (Anderson and Hoffman, 1984). Polycationic ferritin (PCF), was used to show a decrease in negative charge on the surface of the cells at implantation. PCF bound extensively to the uterine epithelial cells of rabbits in estrus and to uteri from ovariectomized females given injections of oil or estradiol. Preincubation of the uteri in neuraminidase or trypsin prior to PCF exposure reduced this labeling considerably. Labeling by day two of pregnancy was limited to small aggregates of PCF distributed along the apical membrane and microvilli. No labeling of the surface was detected on day six of pregnancy, which corresponds to the time of implantation in rabbits. Using a similar technique, Hewitt et al. (1979), also showed a decrease in anionic sites in rat uteri.
Ambiguities in staining techniques make it difficult to distinguish between reduction in charge and clustering of charged groups. Surface charge was unchanged when ruthenium red and thorium dioxide were used as cytological markers, presumably due to inadequate penetration of dyes (Enders, 1974). A novel in vitro assay was developed to show a loss of negative charge at the time of implantation (Morris and Potter, 1984). Uterine epithelial cells were isolated from 3.5 and 4.5-day pregnant mice. Cell aggregates or vesicles were formed after a day in culture. These vesicles were allowed to interact with positively charged DEAE Sephadex (A-50) beads. Nearly 100% of the vesicles from 3.5-day pregnant mice attached to the beads but only 50% of the vesicles from 4.5-day pregnant mice attached. Attachment of the vesicles to the beads was blocked by the addition of dextran sulfate. Attachment was also inhibited when cells were treated with neuraminidase prior to exposure to the beads.

Studies on activated blastocysts also show a decrease in anionic sites at the time of implantation (Jenkinson and Searle, 1977; Nilsson and Hjerten, 1982). Reduction in the negative surface charge during implantation may aid in the adhesion process by reducing the electrostatic repulsion between the epithelial cells of the trophoblast and the uterus (Morris and Potter, 1984). Studies utilizing neuraminidase have suggested that sialic acid is the major source contributing to the negative surface charge on uterine epithelial cells. Thus, the decrease in sialic acid may be an important change in promoting a receptive uterus.
**Uterine Epithelial Surface Glycoconjugates**

Cell surface carbohydrates are major components of the outer surface of mammalian cells. Different levels and types of carbohydrates are expressed during normal cellular events and during differentiation and disease. In many instances, distinct carbohydrates are restricted to specific cell types. Carbohydrates linked to proteins and lipids have been found to play a role in many cell adhesion processes. They are involved in T-cell recognition processes (Carbone and Gleeson, 1997), in fertilization (Wasserman and Wachbroit, 1992) and in early events of the inflammatory response (Lasky, 1992; Varki, 1994). Glycoconjugates have also been implicated in the process of implantation (Kimber, 1994). There is a decrease in the thickness of the glycocalyx, or sugar coat of uterine epithelial cells during implantation (Anderson and Hoffman, 1984; Hewitt et al., 1979). This suggests that the levels of glycoconjugates present at the surface of uterine epithelial cells are lower during this period. Reports have shown that sugar metabolism and glycosylation are stimulated by estrogen and inhibited by progesterone, without affecting the rate of protein synthesis (Carson et al., 1990). This would imply that changes in the composition or complexity of carbohydrates present on existing glycoproteins might play a role in promoting a receptive uterus. This led investigators (Carson et al., 1990) to focus on changes in carbohydrate and glycoconjugate expression at the surface of uterine epithelial cells to identify changes that may be specific to implantation.
**Changes in Carbohydrate Detected by Lectins**

Lectins have been used as a probe to examine changes in carbohydrate levels present in glycoconjugates expressed at the surface of uterine epithelial cells during the period of implantation. Lectins are proteins, which selectively bind to specific carbohydrate structures. They have been used as a valuable tool to isolate and study glycoproteins.

Concanavalin A (Con A) is a lectin that recognizes the neutral carbohydrates glucose and mannose. The level of Con A binding to the surface of epithelial cells is unchanged during early pregnancy in mice (Enders and Schlafke, 1974), sheep (Guillomot et al., 1982) and rabbits (Thie et al., 1986). Anderson et al. (1986), however, detected reduced Con A binding at implantation sites compared to non-implantation sites in rabbits.

*Ricinus communis* agglutinin (RCA-i) recognizes β-linked D-galactose (D-Gal) in a terminal, non-reducing position in the glycocalyx (Nicolson et al., 1974). In mice, RCA-1 binding was not observed during the estrous stage, but elevated binding was observed on the fourth day of pregnancy (Chavez and Anderson, 1985). An increase in RCA-1 binding was also observed in pregnant and pseudopregnant rabbits. Probing transblots of detergent extracts of rabbit uterine epithelium with RCA-1 revealed a 42kd glycoprotein (GP42) that was present in pregnant but not estrous uterine cell extracts (Anderson et al., 1986). Using a GP42 antibody, the label was confined to the apical surface of uterine epithelial cells but not to other cell
types of the uterus. In addition, binding was present in sections of the pseudopregnant and pregnant uterus at the time of implantation, but not in uterine sections obtained from rabbits in estrus. No difference was seen in the staining pattern between pregnant and pseudopregnant uterine sections, indicating that the presence of a blastocyst is not necessary. Hoffman et al. (1996) concluded that GP42 could be a marker of receptivity in the rabbit uterus. Neuraminidase pretreatment of uteri from estrous animals prior to RCA-1 exposure resulted in an increase in lectin binding (Anderson et al., 1986; Chavez and Anderson, 1985). Removal of sialic acid exposes the penultimate sugar, galactose, which then can be recognized by RCA-1.

Several other lectins were used to examine the glycocalyx of uterine epithelial cells in rabbits, but differences were not seen between estrous or pseudopregnant animals (Anderson et al., 1986). These lectins (sugar specificities included in parentheses) included wheat germ agglutinin (N-acetyl glucosamine and sialic acid), soybean agglutinin (N-acetyl galactosamine or D-galactose), and ulex europaeus (L-fucose). A difference in wheat germ agglutinin (WGA) binding, however, was seen in uteri from mice during early pregnancy. WGA staining was most intense on day one of pregnancy and gradually decreased through day five of pregnancy (Surveyor et al., 1995).

Although these studies demonstrated species differences in several carbohydrates, changes in the level of terminal galactose was significant and common to all species studied. The level of galactose was higher when the uterus
was in the receptive state. Therefore, exposed galactose residues may be serving as a recognition molecule for blastocyst attachment. The increase in the level of terminal galactose may reflect a concomitant decrease in sialic acids, because galactose is one of the penultimate sugars to which are bound sialic acid residues. The inverse relationship between WGA and RCA-I levels in mice therefore suggest that sialic acid levels are decreasing. Whether sialic acid is being physically removed or whether a down-regulation in expression is occurring is still an issue that needs investigation.

**Glycoprotein Changes at the Uterine Epithelium Surface**

As another approach, the presence or levels of glycoconjugates have also been studied with specific antibodies. These include growth factors, mucins and glycoconjugates that contain a Lacto-N-Fucopentaose (LNF-1) epitope. In vitro studies show that these glycoconjugates may play a role in implantation (DeSouza et al., 1998; Lindenberg et al., 1988 and Raab et al., 1996).

Frozen sections of mouse uteri from various stages of pregnancy were probed with an antibody that recognizes glycoconjugates containing the LNF-1 oligosaccharide structure. The sugar determinant, LNF-1, is composed of fucose linked to a Galβ1-3GlcNAcβ1 backbone. Binding is present on the surface of luminal and glandular epithelial cells during peri-implantation in a punctate manner on each cell. By day six of pregnancy, the day following implantation, no binding was detected (Lindenberg et al., 1988). A similar binding pattern was also seen on
uterine epithelial cells in pseudopregnant mice, which suggests that this epitope is not dependent on the presence of the blastocyst. The presence of LNF-1 was stimulated by estrogen (Kimber and Lindenberg, 1990). Using an in vitro model system, blastocyst binding to a monolayer of mouse uterine epithelial cells was inhibited by the addition of free LNF-1. A monoclonal antibody that recognizes the LNF-1 epitope also blocked blastocyst attachment (Lindenberg et al., 1988).

Examination of mouse blastocysts showed that an LNF-1 specific receptor was expressed on the abembryonic mural trophectoderm, which is where initial contact is made with the luminal epithelial cells (Lindenberg et al., 1990; Yamagata and Yamazaki, 1991). From these findings, investigators have suggested that glycoconjugates containing the LNF-1 epitope may be involved in initial adhesion of the mouse embryo. The absence of LNF-1 epitope on the uterine cell surface by day six of pregnancy correlates with the refractory period, when the uterus is no longer receptive to blastocyst implantation.

Das et al. (1994) have shown that a heparin binding epithelial growth factor (HB-EGF) is expressed in mouse uterine luminal epithelium six to seven hours prior to the attachment reaction. HB-EGF is a member of the EGF family of growth factors and it can bind to heparan sulfate proteoglycans (HSPG) and EGF receptors. Activated mouse blastocysts contain HSPG (Carson et al., 1993) and EGF receptors (Paria et al., 1993) on their surface. In fact, synthesis of HSPG increases about four to five fold at the peri-implantation period (Smith et al., 1997). HB-EGF on the surface of uterine epithelial cells may interact with HSPG or EGF receptors on the
surface of the blastocyst in the initial stages of implantation. An in vitro model system was used to demonstrate the adhesion properties of HB-EGF (Raab et al., 1996). In this model, HB-EGF constructs were expressed in a mouse cell line and incubated with activated blastocysts. The number of adherent cells observed with electron microscopy was found to be statistically significant.

Inhibition of HSPG synthesis also inhibits the attachment of the embryo to primary uterine epithelial cells in culture (Smith et al., 1997). Studies also show that HB-EGF can induce EGF receptor phosphorylation and promote blastocyst growth and zona hatching (Das et al., 1994). Other members of the EGF family, epiregulin, betacellulin, and amphiregulin are also expressed in uterine epithelial cells at the time of implantation in the mouse (Das et al., 1995; Das et al., 1997) and their expression is limited only to sites of blastocyst implantation. Like HB-EGF, they may be involved in blastocyst adhesion or act as signal transducers to promote blastocyst growth and development.

Muc-1 is another glycoprotein that exhibits changes in expression in uterine epithelial cells. Muc-1 is a large membrane bound glycoprotein ranging between 250-500 kilodaltons. Like other mucins, it is extensively glycosylated, with many sialic acid residues, resulting in high net negative charge on the molecule. The extracellular portion of Muc-1 extends up to 500 nm from the luminal surface, much farther than many other components of the apical glycocalyx. Muc-1 expression is significantly decreased in uterine epithelial cells at the time of implantation in the
mouse (Braga and Gendler, 1993) and rat (DeSouza et al., 1998). In rabbits
(Hoffman et al., 1998), Muc-1 expression decreases only at implantation sites. Muc-
1 expression in humans persists during the receptive state (Hey et al., 1994), but it is
not known if it is reduced locally as in rabbits. It is known, however, that variant
Muc-1 glycoforms are present in the human endometrium in a cycle dependent
fashion (DeLoia et al., 1998). Muc-1 expression is suggested to be under the control
of the steroid hormones estrogen and progesterone. The levels of Muc-1 in uterine
epithelial cells of cycling mice is highest when estrogen levels are high, during
proestrus and estrus (Braga and Gendler, 1993). Estrogen stimulates Muc-1
expression, while progesterone antagonizes the effects of estrogen in rats (Surveyor
et al., 1995).

In vitro studies using polarized uterine epithelial cells indicate that mucins
function to prevent embryo attachment (DeSouza et al., 1998). Polarized uterine
epithelia in culture are usually non-receptive (Julian et al., 1992) and express high
levels of mucins (Pimental et al., 1996). Experimental evidence that mucins
functions to prevent attachment are three fold. First, when mucins are enzymatically
removed from the apical cell surface of polarized epithelia, the cells are converted
from a non-receptive to a receptive state. Second, uterine epithelia from Muc-1 null
mice have an increased capacity to bind embryos in vitro. Third, cells transfected
with Muc-1 bind to mouse blastocysts less efficiently than Muc-1 non-expressing
cells. From these studies, it appears that Muc-1 is acting as an anti-adhesive
From the examples given above, it is clear that there is a change of glycoconjugates present at the surface of uterine epithelial cells. Growth factors and glycoconjugates containing the LNF-1 epitope seem to play adhesive roles during the initial phases of implantation, and Muc-1, an anti-adhesive role during the non-receptive phase. The exact nature of the carbohydrate complex of Muc-1 carbohydrate structures expressed between the non-receptive and receptive luminal epithelium are still not known. However, it is known that one of the major Muc-1 glycoforms found in humans during the receptive period lacks sialic acids (DeLoia et al., 1998). The presence of terminal sialic acid residues may thus contribute to the anti-adhesive properties of Muc-1 by electrostatic repulsion or by steric hindrance during the non-receptive period (Ligtenberg et al., 1992).

SIALIC ACIDS

Sialic acids are acidic monosaccharides that contribute significantly to the variability of cell surface glycoconjugates. They occur mainly as terminal sugars in different linkages bound to a variety of oligosaccharide glycans, which are part of both glycoproteins and glycolipids. Altered levels and types of sialylation occur in different cell types during normal development and in pathological states. It is believed that these changes are related to the role sialic acids play in cell-cell interactions. Sialic acids are shown to play major roles in cellular interactions by
acting as an integral component of a glycan ligand for specific cell adhesion molecules (Crocker et al., 1994; Owens and Bunge, 1989; Powell et al., 1993). Sialic acids also act to inhibit adhesion by masking subterminal recognition structures (Drickamer, 1991; Shimamura et al., 1994). When present in large numbers, sialic acids may also inhibit adhesion by preventing cellular interactions through electrostatic repulsion and stearic effects (Yang et al., 1994).

Sialic acids make up a family of nine-carbon acid sugars, all of which are derivatives of neuraminic acid (Fig. 3). Although N-acetylneuraminic acid is the most abundant sialic acid, more than forty different structural modifications of this sugar have been found in nature (Schauer, 1982; Varki, 1997). Sialic acids are usually terminal sugars that are linked to the terminal, non-reducing positions of oligosaccharide chains. They are bound to core oligosaccharide glycans through different linkages. These include α2,3 and α2,6 glycosidic linkages. Although sialic acids are most often linked to galactose by α2,3 or α2,6-linkages, they are also linked to the 6-hydroxyl group of N-acetylgalactosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) residues. Sialic acids can also be found linked to each other by α2,8 bonds and repetition of this binding type can form long homopolymer chains known as polysialic acid (PSA). Because of its structural modifications and terminal linkage types, sialic acid adds to the variability of cell surface glycoconjugates that allows it to play a major role in cellular interactions.
The most common form of sialic acid is N-Acetylneuraminic acid, where $R_1 = \text{acetyl}$. N-Glycolyneuraminic acid is another form of sialic acid, where $R_1 = \text{glycolyl}$. Numerous other forms of sialic acid is made possible with different O- and N-substituents on carbons 4, 7, 8 and 9 (Schauer, 1982).
Altered levels of sialylation have been found in cells during normal cell processes and in pathological states. An increase in sialylation of cell surface glycoconjugates has been demonstrated in malignant tumors and is correlated with the invasive and metastatic growth of colon carcinoma cells (Sata et al., 1991). Levels of sialylation may also be a mechanism to regulate sensitivity towards apoptotic cell death. Apo-1 is a glycosylated cell surface receptor that mediates apoptosis. The glycan portion of Apo-1 is differentially sialylated. Induction of apoptosis by Apo-1 was found more effective when the receptor contained low levels of sialic acid (Peter et al., 1995). Cell surface sialylation also regulates the maturation and migration of thymocytes in the thymus. Mature thymocytes contain sialoglycoconjugates that immature thymocytes lack. It has been proposed that sialylation in mature thymocytes inhibits the interaction of thymocytes with cortical epithelium and allows these mature cells to migrate to the medulla of the thymus (Gillespie et al., 1993).

Glycoconjugates containing sialic acid in specific linkages act as ligands in cell adhesion events (Crocker et al., 1991; Powell et al., 1993; Varki, 1997). Selectins, are receptors involved in leukocyte trafficking during inflammation, that recognize sialyl Lewis\(^x\), a tetrasaccharide, which contains sialic acid in an $\alpha2,3$ linkage, Neu5Ac$\alpha2,3$Gal$\beta1-4$(Fuc$\alpha1-3$)GlcNAc-R (Berg et al., 1991). Sialoadhesin, CD22, and myelin-associated glycoprotein (MAG) are sialic acid dependent adhesion molecules of the immunoglobin superfamily (Kelm et al., 1994). Sialoadhesins are
restricted to macrophages and mediate interactions with developing myeloid cells in the bone marrow and lymphocytes in spleen and peripheral lymph nodes. Sialoadhesins on the surface of bone marrow macrophages binds to specific ligands on developing myeloid cells that contain α2,3-linked sialic acids (Crocker et al., 1994). CD22 is a B-cell specific molecule which binds to ligands on B-lymphocytes that contain α2,6-linked sialic acids (Powell et al., 1993). MAG is expressed only on myelinating oligodendrocytes and Schwann cells. It plays a crucial role in the early steps of myelination (Owens and Bunge, 1989) and in maintaining the organization of myelinated axons (Li et al., 1994). MAG recognizes specific ligands on axons that contain α2,3-linked sialic acids. These are just a few examples that demonstrate the significance of specific linkages of sialic acid residues involved in cell adhesion.

An opposite role for sialic acids is the inhibition of cellular interactions by masking recognition sites or by preventing receptor-ligand interactions through electrostatic repulsion and steric hindrance. An example of this inhibition is shown by an in vitro assay performed to test the effects sialic acids have on the ability of cells to adhere to plastic and collagen coated surfaces (Shimamura et al., 1994). Swiss 3T3 cells (a fibroblastic cell line) and TES-1 cells (an epithelial cell line) were used as representative adherent cells. Binding of these cells to the substratum was enhanced when sialic acids were removed with neuraminidase (Shimamura et al., 1994). The implication is that sialic acids are apparently masking molecules necessary for the adhesion of these cells. The masking effect of sialic acid is thought
to protect certain serum glycoproteins from being cleared from the blood stream. If sialic acid is removed, the penultimate sugar galactose is exposed and the glycoproteins are readily recognized by asialoglycoprotein receptors. This leads to the eventual clearance of these serum glycoproteins from circulation (Drickamer, 1991).

The neural cell adhesion molecule, NCAM, is negatively regulated by sialic acid. PSA is attached to the glycan portion of NCAM. The presence of long PSA residues increases the distance between opposing cell membranes by more than 10 nm (Yang et al., 1992). PSA also has a high density of negative charge and the capability to surround itself with water molecules. These characteristics allow PSA to produce a physical barrier between cellular interactions by both electrostatic repulsion and steric hindrance through its size, length, charge and hydration effects. Cell surface mucins have also been shown to strongly reduce cell adhesion by acting as a physical barrier and by charge repulsion due to the large sialic acid component (Hilkens et al., 1995; Kemperman et al., 1994; Ligtenberg et al., 1992).

The indirect studies cited earlier have suggested that there is a significant decrease in sialic acids at the apical surface of uterine epithelial cells at the time of implantation. A possible reason for this decrease is the removal of sialic acid, which could relieve its masking or steric effects. The decline of sialic acid would then expose potential adhesion molecules or ligands that might be involved in blastocyst attachment. More direct studies examining the expression of sialic acid residues at
the surface of uterine epithelial cells are needed. More importantly, information on the factors that control the expression of sialic acids will result in a better understanding of the role sialic acids play in the process of implantation. A thorough study of regulation requires that one look not only at the end product, sialic acid, but into the expression and activity of sialyltransferases, the enzymes responsible for the attachment of sialic acids on to glycoconjugates.

GLYCOSYLTRANSFERASES

Structural diversity enables carbohydrates to be important players in cell recognition events. Unlike proteins and nucleic acids, carbohydrates are linked together at more than one site. A sugar residue can be attached to any of three or four hydroxyl groups of neighboring sugar residues. Furthermore, residues can be attached as either α- or β-linkages. These variations allow carbohydrates to have a complicated structure including branches. Because of this complexity, carbohydrates can exist as a vast array of different structures.

The enzymes that carry out biosynthesis of oligosaccharides are called glycosyltransferases. They operate in an assembly-line fashion within the Golgi apparatus, sequentially adding carbohydrate monomers to growing oligosaccharide chains. Due to recent advances in carbohydrate research, we now know more about glycosyltransferases and their specificity, structure, and location within cells. Changes in expression and activity of specific glycosyltransferases results in
differential glycoconjugate expression in cells during development, differentiation and disease (Baum et al., 1996; Jones et al., 1996; Sata et al., 1991).

Glycosyltransferases catalyze the addition of sugars to the carbohydrate portion of glycoproteins and glycolipids. They are specific for their donor substrate (nucleotide sugars) and acceptor substrate (growing carbohydrate group). Each glycosyltransferase is also specific for a particular glycosidic linkage. For example, α2,6-sialyltransferase, one member of the glycosyltransferase family, will form a 2,6-glycosidic linkage between sialic acid and galactose. The specificity of these enzymes for their donor and acceptor substrates is the primary basis for the precision in building complex sugar chains present on glycoproteins and glycolipids within a cell (Schacher, 1994). Glycosyltransferases are grouped into families depending on the type of sugar they transfer. In addition to sialyltransferases, at least three other gene families are known, including galactosyltransferases, glucosaminyltransferases, and fucosyltransferases (Joziasse, 1992).

It is predicted that more than a hundred glycosyltransferases are required for the synthesis of the known carbohydrate structures on glycoproteins and glycolipids, but only a few have been cloned (Schacher, 1994). From the amino acid sequence of the enzymes cloned, a characteristic topology of glycosyltransferases has been predicted (Paulson and Colley, 1989; Schachter, 1991). They all have short NH₂-terminal cytoplasmic tail, a signal anchor domain, a stem region and a large COOH-terminal catalytic domain. The signal anchor domain spans the transmembrane
region and appears to be essential for initial targeting of the enzyme to the Golgi apparatus. The short stem region is believed to serve as a flexible tether for the catalytic domain, which resides in the Golgi lumen.

Although glycosyltransferases are normally found associated with the Golgi membrane, they have also been found as a soluble form and in the plasma membrane. Lammers and Jamieson (1989) found soluble forms of α2,6-ST in serum in response to induced inflammation. Galactosyltransferases have been localized immunocytochemically on the plasma membrane of many different cell types (Pierce et al., 1980). The finding of surface enzymes has stimulated hypotheses involving these enzymes in direct cell-cell recognition events. Galactosyltransferases present on the cell surface are believed to function as a type of lectin, binding to an acceptor molecule present on the cell membrane of an adjacent cell (Shur, 1991; Strous, 1986). Much evidence has been collected over the years to implicate this mechanism in a variety of cell adhesion events including cell migration, fertilization, cell differentiation and the immune reaction (Shur, 1991).

Sialyltransferases are the enzymes that transfer sialic acids to carbohydrate groups of glycolipids and glycoproteins. Thus far, eighteen different sialyltransferase enzymes have been identified (Harduin-Lepers et al., 1995), and most of these have been cloned. Since sialyltransferases share the same sugar donor (CMP-NeuAc), and recognize similar acceptor substrates, it was expected that they would exhibit similar protein sequences. Surprisingly, amino acid sequences of the cloned sialyltransferase
cDNAs show very little homology with the exception of a short consensus sequence called the sialylmotif (Datta and Paulson, 1995) (Fig. 4). Results from mutagenesis studies suggest that the conserved sialylmotif in the sialyltransferase gene family encodes the binding domain of the common donor substrate, CMP-NeuAc (Datta and Paulson, 1995).
Figure 4: The α2,6-sialyltransferase sialylmotif domain.

A.

alpha-2,6 Sialyltransferase Coding Region
Sialylmotif domain

RATGASB KC
1477 bp

B.

+3 nArgCysAla ValValSerSer AlaGlySer LeuLysAsn SerGlnLeuG
501 AAGGTGTGCC GTCTCTTCTT CTGCAGGATC TCTGAAAAC TCCAGCTTG
TTCCACACGG CAGCAGAGAA GACGTCCTAG AGACTTTTTG AGGGTCGAAC

+3 lyArgGluIle AspAsnHis AspAlaValLeu ArgPheAsn GlyAlaPro
601 GTGAGAGAT TGATAATCAT GATGCAGTTC TGAGGTTTAA TGGGGCCCCT
CACCTCTCTA ACTATTAGTA CTACGTCAAG ACACCCAGGTA

+3 ThrAspAsnPhe GlnGlnAsp ValGlySer LysThrThr
651 ACCGACAACT TCCAACAGGA TGTTGGCTCA AAAACTACC
TGGCTGTTGA AGGTTGTCTT ACACCCAGGT TTTTGATGG

The α2,6-sialyltransferase sialylmotif domain is a highly conserved region that is shared among members of the sialyltransferase gene family. It encodes the binding domain of the donor substrate. Panel A shows the position of the sialylmotif domain in purple. The sialyltransferase coding region is shown in red. Panel B shows the amino acid and cDNA sequence for the sialylmotif domain (Datta and Paulson, 1995).
The enzyme β-galactoside α2,6-sialyltransferase produces the NeuAcα2-6Galβ1-4GlcNAc terminus in various N-glycans and some O-glycans (Harduin-Lepers et al., 1995). It was first isolated from rat liver (Weinstein et al., 1982) and has since been isolated from other tissues (Grundmann et al., 1990). In rat liver hepatocytes, this enzyme has been localized by immunoelectron microscopy to the trans-cisternae of the Golgi and the trans-Golgi network. In intestinal absorptive cells, the enzyme is more diffusely localized throughout the cisternal stacks (Taatjes and Roth, 1988). Soluble forms of this enzyme have also been found in various secretions and body fluids including milk and colostrom (Paulson et al., 1977) and in serum (Lammers and Jamieson, 1989). The release of the enzyme from the Golgi membrane is believed to be due to the action of a cathepsin D-like protease on the stem region of the α2,6-ST enzyme. The transcription of the α2,6-ST gene is regulated by multiple promoters and expression is influenced by glucocorticoids and cytokines (Kolinska et al., 1990; Kolinska et al., 1990). The expression of α2,6-ST has been followed in various rat and human tissues. Levels of expression appear to correlate well with the presence of α2,6-linked sialylglycoconjugates present in the respective tissues (Kaneko et al., 1995). In addition, expression is differentially regulated according to tissue type (Kitagawa and Paulson, 1994).

Oligosaccharides have been recognized for their role in cellular interactions in many different biological systems. It is not surprising therefore, that interest in the
enzymes responsible for the biosynthesis of these oligosaccharides is increasing. Much information on the physical structure, cellular location and expression of specific glycosyltransferases is accumulating. However, no information is available on the expression levels of α2,6-ST in uterine epithelial cells. Following the expression levels of α2,6-ST in uterine epithelial cells during peri-implantation will provide insights into the control of α2,6-linked sialic acid levels present during this period.

**SUMMARY**

The initial stage of implantation involves recognition and attachment of the apical plasma membrane of the trophoblast to the apical plasma membrane of the uterine epithelium. Uterine epithelial cells are usually non-adhesive. Therefore, to allow blastocyst attachment, changes must take place at the apical surface of these cells. One change reported is a reduction in the thickness and composition of the glycocalyx of uterine epithelial cells. A second change is a reduction in the negative surface charge. It has been suggested that these changes could be due in part to a decrease in the expression of sialoglycoconjugates.

To date, no direct studies of the changes in α2,6-linked sialylglycoconjugates during the implantation period in uterine epithelial cells exists. In this study, Sambucus nigra (SNA), a lectin, which binds specifically to glycoconjugates containing α2,6-linked sialic acids, was used to examine the levels and localization
of the expression of these glycoconjugates. Expression was followed in the uterine epithelial cells of mice in different stages of the estrous cycle, during early pregnancy, and in ovariectomized mice given hormone replacement. In addition, expression of α2,6-ST, the enzyme responsible for synthesis of sialic acids in α2,6-glycosidic linkages, was followed through these stages.

Results show that the levels of α2,6-linked sialoglycoconjugates decreases at the time of implantation. This decrease correlates with the decrease in expression of the enzyme, α2,6-ST. Levels of α2,6-ST and α2,6-linked sialoglycoconjugates appear to be stimulated by the steroid hormone estradiol and inhibited by progesterone.
CHAPTER 2

MATERIALS AND METHODS

MATERIALS

Calcium magnesium free Hank’s balanced salt solution (CMF-HBSS) was purchased from Gibco-BRL (Grand Island, NY). DC protein assay kit, nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and all compounds used in polyacrylamide gel electrophoresis were obtained from Bio Rad (Hercules, CA). Ultrapure phenol and formamide were from Bethesda Research Laboratories (Gaithersburg, MD). Antibodies and horseradish peroxidase (HRP) conjugated avidin-D were purchased from Vector Laboratories (Burlingame, CA). Leupeptin, pepstatin A, neuraminidase, SNA and the Genius in vitro translation and detection kits were purchased from Boehringer Mannheim (Indianapolis, IN). Enhanced chemiluminescence (ECL) reagents and hyperfilm were from Amersham (Arlington Heights, IL). Immobilon-P nylon membranes were from Millipore (Bedford, MA) and centricon-10 microconcentrators were from Amicon (Bedford, MA). Restriction endonucleases were purchased from New England Biolabs (Beverly, MA). All other chemicals of reagent grade or better were purchased from Sigma Chemical Co. (St Louis, MO).
ANIMALS

CF-1 strain mice were purchased from Charles River Laboratories (Wilmington, MA) and maintained by Oregon State University Laboratory Animal Resources according to the guidelines of the National Institutes of Health. The mice were maintained under 12:12 hours light:dark photocycle and food and water were supplied ad libitum.

Vaginal smears were used to stage the estrous cycle of individual females by the type and appearance of cells in the smears (Martin, 1985). The following guidelines were used: 1) Proestrous: equal numbers of leukocytes and small, round, nucleated epithelial cells; 2) estrous: large, flattened anucleated epithelial cell remnants with irregular and folded edges; 3) metestrous: equal numbers of leukocytes and epithelial cells of the morphology seen in the estrous stage; 4) diestrous: predominately leukocytes (Fig. 5). At least two complete cycles were followed before the mice were used in experiments. To obtain pregnant mice, female mice in estrus were housed individually with a single male of the same strain. Pregnancy was determined by the appearance of a vaginal plug the following morning, which is referred to as day one of pregnancy. The mice were sacrificed by cervical dislocation at day one through day six of pregnancy and at each stage of the estrous cycle. The uteri were removed and cleaned of all fat and mesentery, and prepared for luminal epithelial cell isolation or histochemistry. Female mice undergoing ovariectomy in the lab were given approximately 1.5 ng sodium pentabarbital and supplemented with halothane when necessary. Surgery was
performed and animals were allowed to recover under a heat lamp for approximately five hours before being returned to the animal room. These mice were housed for ten days to allow endogenous steroidal hormones to subside before administering intraperitoneal (i.p.) injections of estradiol and progesterone. Animals were given i.p. injections of saline (vehicle controls), 0.125 μg/mouse 17β estradiol (E2) or 100 μg/mouse progesterone (P4) for three consecutive days. Approximately 20 hours after the last injection, the animals were sacrificed by cervical dislocation and the uterine horns dissected, cleaned of all fat and mesentery, and prepared for histochemistry.
Stages of the estrous cycle were determined by the type and appearance of cells in vaginal smears. (A) diestrus: limited mostly to leukocytes; (B) proestrus: equal number of leukocytes and small, round, nucleated epithelial cells; (C) estrus: large, flattened anucleated epithelial cells with irregular and folded edges; and (D) metestrus: equal numbers of leukocytes and epithelial cells of the morphology seen during the estrus stage. Bar = 10µM.
ISOLATION OF UTERINE EPITHELIAL CELLS

A modification of the method used by White and Kimber (1994), was used to remove epithelial cells from the uterus. Uterine tissue was isolated from mice at each stage of the estrous cycle and on days one through six of pregnancy. Uteri were cut into three to four mm size segments, then placed in calcium magnesium free Hanks’ Balanced Salt Solution (CMF-HBSS) buffered with 0.01 M 3-[N-morpholino]propanesulfonic acid (MOPS), pH 7.3 containing 0.5% dispase and incubated for three hours at room temperature on a rotary shaker. The uterine segments were removed and placed in CMF-HBSS plus 0.4% bovine serum albumin (BSA). To expel the epithelial lining, medium was washed gently through each uterine segment using a pipet with a bore diameter slightly smaller than the uterine horn. Epithelial fragments were collected in a 15 ml conical centrifuge tube placed on ice until epithelial fragments were collected from all uterine segments (no longer than five minutes). Large epithelial fragments were collected at the bottom of the centrifuge tube using a hand powered centrifuge at slow speeds. The supernatant, which contained single cells, was discarded. The fragments were washed with two changes of CMF-HBSS.

EXTRACTION OF UTERINE EPITHELIAL FRAGMENTS

After isolation, epithelial fragments were placed in 4 M Guanidine-HCl and 0.5% Triton X-100 in 0.01 M sodium acetate, pH 5.8. Protease inhibitors (1 mM EDTA, 1 mM leupeptin, 0.2 mM phenylmethylsulfonylefluoride, and 0.1 mM
pepstatin A) were added to the extraction buffer (Potter et al., 1996). Samples were extracted overnight on a rocking shaker at 4°C. Extracts were run through Centricon 10 microconcentrators to replace the extraction buffer with 0.1 M Tris-HCl, pH 7.5, containing 0.15 M NaCl and protease inhibitors.

**SDS-PAGE AND LECTIN TRANSBLOTS**

Prior to running samples on gels, protein concentrations were determined using the Bio-Rad DC Protein Assay. Samples were normalized by dilution to ensure equal protein concentrations were used. Samples were heated for five minutes in SDS sample buffer (2% SDS, 5% β-mercaptoethanol, 10% glycerol in 50 mM Tris-HCl, pH 6.8) and loaded on 5%-15% gradient SDS-polyacrylamide gels using the method described by Laemmli (1970). Pre-stained standards were used to determine protein molecular weights. Proteins were transferred from the gels (Towbin et al., 1979) to Immobilon-P PVDF membranes using a Genie transblot apparatus (Idea Scientific). To verify equal loading of protein, blots were stained with 1% Amido black in 50 mM Tris-HCl, pH 7.5 containing 0.15 M NaCl (TBS) for one minute and destained with water prior to probing with SNA. When equal protein load was confirmed, the blot was blocked for non-specific sites in 5% BSA fraction V pre-treated with periodic acid for one hour at room temperature. The blots were rinsed in three changes of TBS containing 0.1% Tween-20 (TBST), then incubated in 1 μg biotin-conjugated *Sambucus nigra* (biotin-SNA) per ml TBS containing 1 mM CaCl$_2$ (TBS/Ca$^{2+}$) for one hour at room temperature or overnight at 4°C. Blots were
washed three times with TBST/Ca\(^{2+}\) for ten minutes each, followed by incubation in 1 \(\mu g/ml\) streptavidin-HRP in TBST/Ca\(^{2+}\) for one hour at room temperature. After rinsing with TBST/Ca\(^{2+}\), protein bands containing \(\alpha\)2,6-linked sialic acid residues were visualized using enhanced chemiluminescence (ECL). Hyperfilm was exposed for one to five minutes to obtain optimal signal. As a negative control, duplicate membranes were incubated with 0.1U neuraminidase from *Vibrio cholera* in 50 mM sodium acetate, pH 5.5 overnight at 37°C prior to incubation with SNA.

**LECTIN HISTOCHEMISTRY**

The uterine horn was cut transversely into 50 mm size pieces and embedded in OCT (Miles, Elkart, IN), frozen immediately in liquid nitrogen, and stored at -80°C until use. Cross-sections of six to eight \(\mu m\) were made at -20°C and placed on chrome alum gelatin coated slides to promote adherence. Sections were air dried for ten minutes then placed in 100% methanol containing 0.5% (v/v) hydrogen peroxide for 30 minutes to inactivate endogenous peroxide activity. After rinsing in TBS, sections were incubated in 1 \(\mu g/ml\) biotin-SNA in TBS/Ca\(^{2+}\) for one hour at room temperature or overnight at 4°C. The sections were washed three times with TBS/Ca\(^{2+}\) for 15 minutes each wash with gentle shaking followed by incubation in 1 \(\mu g/ml\) streptavidin-HRP in TBS/Ca\(^{2+}\) for one hour at room temperature. The sections were washed with three changes of TBST/Ca\(^{2+}\) for 15 minutes. Sites containing \(\alpha\)2,6-linked sialic acid residues were detected using 0.05% (w/v)
diaminobenzidine tetrahydrochloride dihydrate in TBS with 0.015% (v/v) hydrogen peroxide (DAB solution) for five to ten minutes at room temperature. Sections were then washed in water and mounted in glycerol. As a negative control, sections were incubated in TBS/Ca²⁺ instead of SNA. Sections were also treated with 0.1U neuraminidase in 50 mM sodium acetate, pH 5.5 overnight at 37° C before probing with SNA.

**IMMUNOHISTOCHEMISTRY**

Sections were prepared as described above but were probed with an affinity purified rabbit polyclonal antibody that recognizes α2,6-ST (a generous gift from Dr. Karen Colley, University of Illinois College of Medicine, Chicago, Illinois). Sections were incubated in a 1:100 dilution of the antibody in PBS for either one hour at room temperature or overnight at 4°C. After washing the sections in three changes of PBST over a period of 30 minutes, sections were incubated in goat anti-rabbit IgG conjugated to horseradish peroxidase for one hour at 4°C. Sections were washed before placing in DAB solution for five to ten minutes at room temperature to detect sites containing α2,6-ST. Sections were rinsed in water before mounting in glycerol. As a negative control, sections were incubated in PBS instead of α2,6-ST antibody.
IN SITU HYBRIDIZATION

Preparation of RNA Probe

The cDNA encoding α2,6-ST, cloned into an EcoRI site of pBluescript II K/S+ (α2,6-ST/BSKS+), was a generous gift from Dr. Karen Colley, University of Illinois College of Medicine, Chicago, Illinois (Fig. 6). It was amplified to provide sufficient material for the preparation of an RNA probe. MV1190 bacterial cells were made competent by the calcium chloride procedure (Maniatis et al., 1982) and transformed with α2,6-ST/BSKS+. Transformed cells were selected on ampicillin plates (100 µg/ml) overnight. Twelve clones were picked, and minipreps analyzed by the alkaline lysis mini plasmid preparation method (Maniatis et al., 1982). The plasmid was cleaved with EcoRI and the DNA fragments were analyzed on a 1% agarose gel. Large scale DNA preparations were prepared for the appropriate clones, and purified using Qiagen columns (Qiagen, Inc., Valencia, CA).

Digoxigenin labeled RNA probes were prepared using digoxigenin-UTP and the appropriate viral RNA polymerase according to the manufacturer’s directions. α2,6-ST was transcribed in the antisense orientation restricted with Xcm I from the T3 promoter and in the sense orientation restricted with BstEII from the T7 promoter (Fig. 6). These specific restriction sites were chosen to exclude the sialylmotif region that is a conserved region in all cloned sialyltransferases.
In situ hybridization using the α2,6-ST RNA probes was conducted as
described by the manufacturer. Labeling efficiency was checked by spotting diluted
aliquots of the labelled cRNA probes on nylon membranes. Spots were analyzed
with the digoxigenin (DIG) luminescent detection kit according to the manufacturers
directions.

**Preparation and Pretreatment of Tissue Sections**

Sections of uteri were prepared as described above and placed on chrome
alum gelatin coated slides. After mounting the tissue sections, the slides were
placed on a heating plate set at 37°C for ten minutes to allow sections to dry and to
fix the RNA in the tissue. The sections were then fixed in PBS containing 4%
paraformaldehyde for 30 minutes at room temperature followed by three ten minute
washes in PBST. All of the solutions used here and in the following steps were
prepared with water treated with 0.1% diethylpyrocarbonate (DEPC) to inhibit
potential RNase activity. The tissue sections were sequentially rinsed with methanol
to extract lipids, which minimized non-specific background. Next, the tissue
sections were placed in 0.5% acetic anhydride in 0.1 M triethanolamine buffer, pH
8.0 for ten minutes followed by three five minute washes in PBST. This acetylation
step helps to reduce the background by acetylation positive charges on molecules in
the tissue, thereby reducing non-specific interactions with the negative RNA probe.
The alpha 2,6-ST cDNA (shown in green) was inserted in the EcoRI site of pBluescript II K/S+. The coding region for alpha 2,6-ST is shown in red. The sialylmotif domain is shown in purple. Digoxigenin labeled probes were prepared with digoxigenin-UTP and the appropriate RNA polymerase. Antisense probes were made by cleaving with Xcm I and transcribing from the T3 promotor. Sense probes used as negative controls were made by cleaving with Bst EII and transcribing using the T7 promotor.
Prehybridization, Hybridization and Posthybridization

Sections were prehybridized with unrelated DNA and RNA to block non-specific sites for 60 minutes at 50°C in hybridization buffer. The hybridization buffer contained 50% deionized formamide, 1X Denhardt’s solution (0.02% ficoll, 0.02% polyvinyl pyrolidone, 0.02% BSA), 10% dextran sulfate, 500 ug/ml herring sperm DNA, 250 ug/ml yeast transfer RNA in 4X SSC or 0.60 M NaCl, 0.060 M sodium citrate, pH 7.0 (0.015 M NaCl in 0.015 M sodium citrate, pH 7.0 = 1X SSC). After prehybridization, 500 ul of hybridization buffer containing 200 ng/ml digoxigenin labeled RNA probes were placed over the sections. A siliconized cover slip was placed over the sections and kept in place by sealing the edges with rubber cement. The slides were placed in a humidified chamber and hybridization was allowed to take place for 16 hours at 50°C. After the hybridization step, cover slips were removed by placing slides in a coplin jar filled with 2X SSC. The sections were then placed through stringent washes to remove nonspecifically bound probe. Washes consisted of two 30 minute washes in 2X SSC containing 50% deionized formamide at 50°C, followed by a ten minute wash in 2X SSC.

Immunological Detection

Sections were washed in three changes of TBST, ten minutes each wash to prepare the sections for immunological detection. To block non-specific sites, the sections were placed in TBST containing 2% normal sheep serum for 30 minutes at room temperature. Excess blocking solution was removed and the sections were
incubated overnight at 4°C with a 1:200 polyclonal sheep antidigoxigenin/alkaline phosphatase conjugate in TBST containing 0.2% normal sheep serum. The sections were washed in TBST, followed by three, five minute washes in detection buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, and 50 mM MgCl₂). Hybridization was detected by covering the sections with detection buffer containing 0.18 mg/ml nitroblue tetrazolium (NBT), 0.34 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and 240 µg/ml levamisole. Color reaction was stopped by washing the sections for five minutes in 10 mM Tris (pH 8.0), 1mM EDTA. The sections were washed in distilled H₂O and mounted with glycerol.
CHAPTER 3

RESULTS

LEVELS OF $\alpha2,6$-LINKED SIALIC ACID IN UTERINE EPITHELIAL CELL EXTRACTS

In order for implantation to occur, uterine epithelial cells must undergo changes to become receptive to blastocyst attachment. To examine whether there are changes in the levels of $\alpha2,6$-linked sialic acid specific to uterine epithelial cells, these cells were isolated from the rest of the uterus. A modification of the method used by White and Kimber (1994), was used to isolate clean flakes of uterine epithelial cells (Fig. 7). Extracts of these isolated flakes were run on SDS PAGE and transblotted after normalization to ensure equal protein concentration. To verify even loading and transfer, the membranes were first stained with amido black. The same membrane was then probed with SNA to examine levels of $\alpha2,6$-linked sialic acid contained in uterine epithelial cells. Control experiments were done to see if there was any effect of amido black staining on glycoprotein detection. Blots were probed with SNA with or without prior amido black staining. No differences in the results were observed (data not shown).
Figure 7: Isolated uterine epithelial flakes.

Photomicrograph of epithelial flakes isolated from a mouse in diestrus. After incubation in dispase, the epithelial lining was separated from the rest of the uterus by flushing media through the uterine lumen with a pipet whose bore diameter was slightly smaller than the uterine horn. Large pieces of epithelial flakes were collected, largely free of contamination from other uterine cell types. Bar = 10 μM.
Changes in extracts from mice in early pregnancy

Proteins isolated from uterine epithelium of mice during the early days of pregnancy were separated on SDS-PAGE gels, transblotted, and probed for sialic acid positive bands with biotinylated SNA. There was a change in the general profile of SNA-stained bands detected from uterine epithelial cell extracts from day one to day six of pregnancy. Two of the most prominent bands that decreased in intensity as the period of implantation approached included a 150 kDa band and a broad band between 110 kDa to 135 kDa. Attachment of mouse embryos typically begins on day five and invasion on day six. The 150 kDa band started decreasing on day five and the 110 kDa to 135 kDa broad band began decreasing on day three of pregnancy and was missing on day four. Two bands at 60 kDa and 85 kDa appeared on day four, and started decreasing on day six of pregnancy. When a duplicate blot was pre-treated with neuraminidase before being probed with SNA, the only clearly visible band was a 130 kDa band (Fig. 8). The staining of this band was probably due to a non-specific avidin binding protein since it was also detectable with strepavidin-HRP alone (data not shown). Overall, there is a pronounced change in SNA-labeled glycoproteins in uterine epithelium at the initiation of blastocyst attachment (during day five). Major changes include a reduction in a dominant 110-135 kDa glycoprotein and the appearance of several smaller glycoproteins.
Extracts of uterine luminal epithelial cells from day one through day six pregnant mice were run on SDS-PAGE after adjusting loading for equal protein concentration, and transblotted on to a PVDF membrane. The blot was probed with biotin conjugated SNA followed by avidin-HRP. Bands positive for α2,6-linked sialic acids were detected by chemiluminescence. There was a pronounced change in the profile of SNA-stained bands that occurred just prior to implantation. Initiation of implantation occurs on day five as indicated by the arrow. Major bands that changed during this period are labeled on the left. As a control, a duplicate blot was incubated with 0.1 units of neuraminidase for one hour at 37°C prior to probing with biotin conjugated SNA. Only a single non-specific band at 130 kDa was detected in all lanes. Molecular weight standards (x10^3) are shown on the right.
Changes in extracts from mice during the estrous cycle

To examine whether the appearance of α2,6-linked sialic acids in uterine epithelial cells is also regulated during the estrous cycle, extracts from cycling mice were probed with SNA. The band profile from mice in estrus and metestrus (Fig. 9) look similar to the pattern seen in the extracts from mice on days one and two of pregnancy (Fig. 8). The intensity of the 150 kDa band and the broad band (115 to 135 kDa) were highest during these stages. The intensity of these bands decreased during metestrus and diestrus and was accompanied by the appearance of a 60 kDa and an 85 kDa band, as seen during day four of pregnancy. A duplicate blot was treated with neuraminidase before probing with SNA and only the non-specific band of 130 kDa remained, confirming that the other bands contain α2,6-linked sialic acids (Fig. 9).

Expression of α2,6-linked sialic acids in uterine sections

Initial blastocyst contact is made with the apical surface of uterine epithelial cells. One would expect that changes to the apical surface would have the greatest impact on implantation. To examine whether α2,6-linked sialic acids are expressed on the apical surface of uterine epithelial cells, histological cross-sections of mouse uteri were probed with biotin-SNA. The histological sections analyzed were from the same uteri used in lectin transblot experiments. A portion of each uterus was prepared for histological sectioning and the rest was used for epithelial cell isolation.
and extraction. Uteri from day one through day six pregnant mice and cycling mice were all prepared in this manner. Sections were also prepared from ovariectomized mice given hormone replacement. Unless otherwise specified, uterine sections from ten mice at each stage of the estrous cycle and days one through six of pregnancy were examined. At least six cross-sections from each mouse were probed with SNA. Description of lectin binding will be limited only to the luminal epithelial cells of the uterus.
Extracts of uterine luminal epithelial cells from mice during the different stages of the estrous cycle were run on SDS-PAGE and transblotted on to PVDF membrane. The blot was probed with biotin conjugated SNA followed by avidin-HRP. Bands positive for α2,6-linked sialic acids were detected by chemiluminescence. Major bands that changed during this period are labeled on the left. As a control, a duplicate blot was incubated with 0.1 units neuraminidase for one hour at 37°C prior to probing with SNA. Only a single band at 130kd was detected in all lanes. Molecular weight standards (x 10$^3$) are shown on the right. D=dioestrus, P=proestrus, E=estrus, and M=metestrus.
In Early Pregnancy

Presence of SNA staining was limited only to the apical surface of uterine epithelial cells (Fig. 10). No staining could be detected at the lateral or basal surface of these cells. The staining was most intense at day one of pregnancy and it decreased gradually up to day four. By day five, or at the time of implantation, no SNA staining could be detected, at either implantation and non-implantation sites (Fig. 11). Control sections that were incubated with neuraminidase prior to probing with SNA showed no SNA staining (Fig. 12). This confirms that the staining seen at the apical surface on days one through four of pregnancy was due to specific sialic acid detection and not due to non-specific binding.

During the Estrous Cycle

SNA staining at the apical surface of uterine epithelial cells was stage-dependent. Staining was most intense during estrus and metestrus and less staining was detected during proestrus (Fig. 13). No staining was detected during diestrus and in control sections treated with neuraminidase prior to probing with SNA. Staining, when present, was limited to the apical surface of the cells. The level of α2,6-linked sialic acids was highest following peak levels of estrogen and low to undetectable after peak levels of progesterone. These results suggest that steroid hormones may be involved in regulating the levels of α2,6-linked sialic acids.
Figure 10: α2,6-linked sialic acid in frozen sections of mouse uterus during early pregnancy.

Frozen sections of uteri from day one through day six pregnant mice were probed with biotin-SNA as described in the methods section. Staining was present at the apical surface of the luminal epithelial cells and was most intense on day one of pregnancy (A). Staining decreased gradually through days two (B), three (C), and four (D). No staining was detected on day five (E) and six (F) of pregnancy. Bar = 10 μM.
Figure 11: Implantation site probed with SNA.

(A) Section through an implantation site from a mouse on day six of pregnancy probed with SNA. No staining was detected at either non-implantation (NI) and implantation (I) sites. Bar = 20 μM. Inset (B) shows the implantation site at a higher magnification.
Figure 12: Frozen sections of mouse uteri during early pregnancy treated with neuraminidase prior to probing with SNA.

Frozen sections were treated with 1.0 unit neuraminidase for one hour at 37°C prior to probing with biotin-SNA. No staining was detected at the apical surface of uterine epithelial cells on day one through day six of pregnancy. A through F represents day one through day six respectively. Bar = 10 μM.
In Ovariectomized Mice Given Hormone Replacement

In order to investigate directly whether estrogen and progesterone are involved in the regulation of the expression of α2,6-linked sialic acids, histological sections of uteri from ovariectomized mice given either estradiol, progesterone or vehicle alone were probed with SNA. At least six sections from three mice in each group were examined. Control mice showed only slight staining at the apical surface of uterine epithelial cells. Mice given estradiol alone showed an increase in SNA staining. No staining was detected in uterine sections from mice given progesterone alone. In addition, no staining was detected in control sections that were pre-treated with neuraminidase (Fig. 14).
Frozen sections of mouse uteri from various stages of the estrous cycle were prepared and probed with SNA as described in the methods section. A strong signal at the apical surface of the luminal epithelial cells was seen during estrus (C) and metestrus (D). The signal was less intense during proestrus (B) and was not detected during diestrus (A). Serial sections were treated with neuraminidase prior to probing with SNA and no staining was detected during diestrus (E), proestrus (F), estrus (G), and metestrus (H). Bar = 10 μM.
Frozen sections from ovariectomized mice given hormone replacement were probed with biotin-SNA as described in the methods section. Staining was detected only at the apical surface of uterine epithelial cells from mice injected with estradiol alone (B). Less staining was detected in controls given vehicle alone (A). No staining was detected in sections from mice injected with progesterone (C). Serial sections were treated with neuraminidase prior to probing with SNA and no staining was detected in sections from mice injected with estradiol (E), progesterone (F), or vehicle alone (D). Bar = 10 μM.
EXPRESSION OF $\alpha_{2,6}$-SIALYLTRANSFERASE MRNA IN UTERINE EPITHELIAL CELLS

$\alpha_{2,6}$-sialyltransferase is the enzyme responsible for the formation of an $\alpha_{2,6}$-glycosidic linkage between sialic acid and Gal$\beta$1-4GlcNAc structures. In situ hybridizations on uterine sections were used to examine the relationship between the levels of $\alpha_{2,6}$-linked sialic acid and the expression of $\alpha_{2,6}$-sialyltransferase. Serial sections from the uteri that were used in SNA binding experiments were used for in situ hybridization to allow a direct comparison.

In Early Pregnancy

As shown in Fig. 15, hybridization with an antisense RNA probe for $\alpha_{2,6}$-ST produced staining in epithelial cells on days one and two of pregnancy. The staining appeared uniform throughout the cytoplasm but was notably absent from the nuclei of the cells. By day three, staining significantly decreased and was not detectable on day four through day six of pregnancy. Control sections that were hybridized with a sense orientation probe demonstrated negligible background staining (Fig. 16). Overall, the results show that $\alpha_{2,6}$-ST mRNA was expressed on days one, two, and to a lesser degree, on day three of pregnancy. These results correlate well with the levels of $\alpha_{2,6}$-linked sialic acids detected with SNA on days one through four of pregnancy.
Figure 15: Expression levels of α2,6-ST mRNA in uterine epithelial cells from mice in early pregnancy.

Frozen sections of mouse uteri during early pregnancy were hybridized with an anti-sense digoxigenin labeled α2,6-ST RNA probe as described in the methods section. Staining in the cytoplasm of luminal epithelial cells was most intense on days one (A) and two of pregnancy (B). By day three (C), staining decreased significantly and was not detected on days four (D), five (E), and six (F) of pregnancy. Bar = 10 μM.
Frozen sections of mouse uteri were hybridized with a sense orientation digoxigenin labeled α2,6-ST RNA probe as described in the methods section. No staining was detected on sections from day one through day six of pregnancy, A through F respectively. Bar = 10 μM.
During the Estrous Cycle

Uterine sections from mice in proestrus and estrus showed equal levels of staining in the cytoplasm of epithelial cells after probing with the antisense mRNA probe for α2,6-ST (Fig. 17). There was also staining in the epithelial cells from mice in metestrus, but the staining intensity was reduced. Very little staining was seen in the sections from mice in diestrus and no staining was detected in the sections hybridized with the sense orientation RNA probe for α2,6-ST. The expression of α2,6-ST mRNA was stage-dependent, with highest levels of expression coinciding with elevated estrogen levels in the uterus, and low or undetectable when progesterone dominated.

In Ovariectomized Mice Given Hormone Replacement

To examine directly whether estrogen and progesterone are involved in the expression of α2,6-ST, uterine sections from ovariectomized mice were hybridized with RNA probes for α2,6-ST. Staining was detected in the cytoplasm of epithelial cells from mice treated with estradiol and less staining was detected in control sections from mice given vehicle alone. No staining was detected in the sections from mice given progesterone alone or in sections hybridized with the sense orientation RNA probe for α2,6-ST (Fig. 18). These results suggest that estradiol may have a role in stimulating the expression of α2,6-ST and they correlated well with the results seen in the levels of SNA binding.
Figure 17: Expression levels of α2,6-ST mRNA in uterine epithelial cells from mice during the various stages of the estrous cycle.

Frozen sections of mouse uteri during the various stages of the estrous cycle were hybridized with an anti-sense digoxigenin labeled α2,6-ST RNA probe as described in the methods section. Staining was most intense in the cytoplasm of uterine epithelial cells during proestrus (B) and estrus (C) and it decreased during metestrus (D) and diestrus (A). No staining was detected when the sense orientation probe for α2,6-ST was used during diestrus (E), proestrus (F), estrus (G) and metestrus (H). Bar = 10 μM.
Frozen sections from ovariectomized mice given hormone replacement were hybridized with an anti-sense digoxigenin labeled α2,6-ST RNA probe as described in the methods section. Intense staining was present in uteri from mice injected with estradiol (B). Faint staining was detected in controls given vehicle alone (A) and no staining could be detected in mice injected with progesterone alone (C). No staining was detected when the sense orientation probe for α2,6-ST was used to probe estradiol injected (E), control (D), and progesterone injected mice (F). Bar = 10 μM.
EXPRESSION OF $\alpha$2,6-SIALYLTRANSFERASE PROTEIN IN UTERINE EPITHELIUM

Results from this study indicated that the expression pattern of $\alpha$2,6-ST mRNA and $\alpha$2,6-linked sialic acid correlated well. They both had high levels of expression during days one and two of pregnancy. Levels decreased during days three and four and no expression was detected by day five, which coincides with implantation. However, there was a slight difference in the timing of expression during the estrous cycle. Expression of $\alpha$2,6-ST mRNA was highest during proestrus and estrus, whereas $\alpha$2,6-linked sialic acid levels was highest during estrus and metestrus. This may be due to the time it takes for the message to be translated, followed by the actual synthesis of $\alpha$2,6-linked sialic acids. In order to have a close correlation in the timing of the expression of both $\alpha$2,6-ST mRNA and $\alpha$2,6-linked sialic acids, the message must first be translated into a functional protein. To follow the expression of $\alpha$2,6-ST protein, sections were probed with a polyclonal antibody made against $\alpha$2,6-ST. Expression was followed during the estrous cycle, early pregnancy, and in ovariectomized mice given hormone replacement.

During early pregnancy

After probing sections with a polyclonal antibody against $\alpha$2,6-ST, staining was detected in the cytoplasm of uterine epithelium on days one and two of pregnancy. Staining intensity decreased on days three and four, and very little
staining could be detected on days five and six (Fig. 19). No staining was detected when sections were reacted with secondary antibody alone.

**During the estrous cycle**

The expression of α2,6-ST protein was stage dependent as was the expression of α2,6-ST mRNA and α2,6-linked sialic acids. Highest levels of staining were detected during estrus with less staining during proestrus and metestrus. Staining in the cytoplasm of epithelial cells during diestrus was close to background levels (Fig. 20). No staining was detected when sections were reacted with secondary antibody alone.

**In ovariectomized mice given hormone replacement**

Staining was highest in the cytoplasm of uterine epithelium from mice injected with estradiol. Sections of control mice showed only slight staining and no staining was detected in sections from mice injected with progesterone (Fig. 21). No staining was detected in sections reacted with secondary antibody alone.
Figure 19: Expression of α2,6-ST protein in uterine epithelial cells from mice during early pregnancy.

Frozen sections of mouse uteri during early pregnancy were probed with a polyclonal antibody against α2,6-ST as described in the methods section. Staining in the cytoplasm of uterine luminal epithelial cells was most intense during days one (A) and two (B) of pregnancy and it gradually decreased on days three (C), four (D), five (E), and six (F). Bar = 10 μM.
Figure 20: Expression of α2,6-ST protein in uterine epithelial cells from mice during the estrous cycle.

Frozen sections of mouse uteri during the estrous cycle were probed with a polyclonal antibody against α2,6-ST as described in the methods section. Staining was most intense during estrus (C) and less staining was detected during proestrus (B) and metestrus (D). No staining was detected during diestrus (A). Bar = 10 μM.
Figure 21: Expression of α2,6-ST protein in uterine epithelial cells from ovariectomized mice given hormone replacement.

Frozen sections from ovariectomized mice given hormone replacement were probed with a polyclonal antibody against α2,6-ST as described in the methods section. Staining was detected in the cytoplasm of uterine epithelial cells from mice injected with estradiol (B). Faint staining was detected in controls given vehicle alone (A). No staining could be detected in sections from mice injected with progesterone (C) and in sections of estradiol-injected mice probed only with secondary antibody (D). Bar = 10 μM.
DECREASE IN GLYCOCONJUGATES CONTAINING α2,6-LINKED SIALIC ACIDS AT THE TIME OF IMPLANTATION

In this study, I was able to show a decrease in the levels of glycoconjugates containing α2,6-linked sialic acids at the surface of uterine epithelial cells at the time of implantation. Transblots of uterine epithelial cell extracts taken from day one to day six pregnant mice probed with SNA revealed a change in the profile of α2,6-linked sialic acid positive bands occurring just prior to implantation. Because whole epithelial cells were extracted, and not just the surface, there were several low molecular weight bands that increased in intensity on day four of pregnancy. These bands may represent lysosomal cleavage products of larger sialoglycoproteins. Support for this interpretation is the fact that the same bands appeared in extracts from epithelium in diestrus and proestrus, a period of tissue regression and recovery. Another possible explanation for these bands is stromal cell contamination. Although great care was taken to obtain pure epithelial cell extracts, stromal cell contamination cannot be ruled out, especially during peri-implantation, when epithelial cell isolation is more difficult.
A decrease in $\alpha$2,6-linked sialic acid was clearly observed in uterine sections. The highest levels of $\alpha$2,6-linked sialic acids were found on day one and then gradually decreased up to day four of pregnancy. By SNA staining, no $\alpha$2,6-linked sialic acids were detected on day five at both implantation and non-implantation sites. This suggests that the decrease in $\alpha$2,6-linked sialic acids is not influenced by the blastocyst and that these changes are intrinsic to the uterus. Expression is limited only to the apical membrane surface of uterine epithelial cells. This location is where one would expect changes in $\alpha$2,6-linked sialic acids to have the greatest impact on blastocyst attachment.

This is the first known report showing a decrease in the expression of $\alpha$2,6-linked sialic acids at the surface of uterine epithelial cells at the time of implantation. Other researchers have used lectins to examine levels of sialylation in uterine epithelial cells, but the lectins used either lacked sialic acid specificity or had a broad reactivity towards different sialic acid linkages (Anderson et al., 1986; Surveyor et al., 1995). Wheat germ agglutinin (WGA) is a lectin that is often used for the detection of sialoglycoconjugates, but it also recognizes N-acetylglucosamine. An increase in WGA binding suggests an increase in sialic acids or N-acetylglucosamine levels (Gallagher et al., 1985). Free sialic acid or N-acetylglucosamine can be used as a competing ligand to discriminate against binding to a particular sugar. Although often used as controls, caution must be taken when interpreting results. Binding constants of free sugars with a lectin have often been found to be several orders of
magnitude lower than the binding constants of glycoconjugates containing this sugar (Debray et al., 1981). This is because the monosaccharides in a terminal non-reducing position on a glycan may not be the only moiety being recognized by the lectins. WGA also has a broad reactivity towards different forms and linkages of sialic acid. Sialic acid as discussed earlier, has many derivatives and can occur with different linkages to different sugars (Varki, 1997). Although the expression of one form of sialic acid may increase while another decreases, this change would not be detected with a lectin like WGA. *Limax flavus agglutinin* and limulin from the hemolymph of *Limulus polyphemus* are lectins that are specific for sialic acids (Miller et al., 1982), but like WGA, they lack specificity. They recognize different forms and linkages of sialic acid, and may not reveal linkage-specific sialic acid changes (Knibbs et al., 1991). The advantage of using SNA is that it is specific for sialic acids that are linked in an \(\alpha2,6\)-glycosidic linkage to Gal\(\beta1\)-4GlcNAc or to GalNAc\(\beta1\)-4GlcNAc (Shibuya et al., 1987). Another glycosidic linkage that is common in cells is \(\alpha2,3\), and this linkage can be specifically detected with the lectin *Maakia amurensis* (Knibbs et al., 1991). Both SNA and MAA have been used extensively in recent years to compare the levels of \(\alpha2,3\) and \(\alpha2,6\) sialylation in different cells and tissues. Different levels of \(\alpha2,3\) and \(\alpha2,6\)-linked sialic acid were shown, which would not have been detected with a general sialic acid lectin. Some examples include human and rat tissues (Kaneko et al., 1995); colon carcinoma cells (Sata et al., 1991) and developing T-cells (Baum et al., 1996). Rat and mouse
uterine sections were also probed with SNA to examine changes in α2,6-linked sialic acid expression in decidual cells during implantation (Jones et al., 1996). Unfortunately, no mention was made on staining in luminal epithelial cells, therefore, their results cannot be compared with the results from this study.

Some of the changes reported in uterine epithelial cells at the time of implantation can be attributed to a decrease in α2,6-sialoglycans. A decrease in surface negative charge was reported in mice (Morris and Potter, 1984), rats (Hewitt et al., 1979), and in rabbits (Anderson and Hoffman, 1984). A decrease in α2,6-linked sialic acids would result in a decrease in cell surface negative charge. In addition, decreased thickness of the glycocalyx (Anderson and Hoffman, 1984; Hewitt et al., 1979) could result from a decrease in the number of α2,6-sialoglycoconjugates being expressed. Last, the increased number of terminal galactose residues (Chavez and Anderson, 1985) may be due to the lack of α2,6-linked sialic acids on Galβ1-4GlcNAc structures. Taken together, the decrease in α2,6-linked sialic acids correlates well with earlier reports made on the changes seen at the surface of uterine epithelial cells at the time of implantation.
FACTORS INVOLVED IN THE REGULATION OF THE LEVELS OF α2,6-LINKED SIALIC ACIDS

**Decrease in the levels of α2,6-sialyltransferase**

One objective of this study was to examine the relationship between expression levels of α2,6-ST with the levels of α2,6-linked sialylglycoconjugates. α2,6-ST catalyzes the final reaction in the biosynthesis of α2,6-sialylglycoconjugates. Therefore, one would expect to see the levels of α2,6-ST to correlate with the levels of glycoconjugates that contain α2,6-linked sialic acids. It is generally accepted that the expression of specific glycosyltransferases in a particular cell will determine the types of oligosaccharide structures made by that cell (Kitagawa and Paulson, 1994; Paulson and Colley, 1989; Wang et al., 1990). The results from this study demonstrate a correlation between the expression of α2,6-ST mRNA, its protein product and the levels of α2,6-sialglycoconjugates in uterine epithelial cells.

The levels of both α2,6-ST mRNA and its protein product were highest on days one and two of pregnancy. Expression decreased on day three and was negligible on days four to six. The level of α2,6-sialglycoconjugates was also high on day one and it gradually decreased up to day four (Fig. 22). From these results, the level of α2,6-sialglycoconjugates correlates well with the levels of α2,6-ST.
From this study, the steady state levels of α2,6-ST mRNA appear to decrease at the time of implantation. It cannot be concluded if regulation is limited to a down regulation of transcriptional events, or whether the stability of the existing pool of α2,6-ST mRNA is being affected.

Expression levels of α2,6-sialoglycoconjugates are dependent not only on the expression of α2,6-ST but also on the level of its enzyme activity. An attempt was made to study the activity levels of α2,6-ST using a novel non-radioactive solid phase assay (Mattox et al., 1992), but the signal to noise ratio was too low. To date, no studies of α2,6-ST activity in uterine epithelial cells during early pregnancy appear to have been reported. The activity of α2,3 and α2,8-sialyltransferase was examined in human endometrium during early pregnancy (Zhu et al., 1990). α2,3-Sialyltransferase activity increased and α2,8-Sialyltransferase activity decreased relative to non-pregnant levels.

Post-transcriptional modification of α2,6-ST has been found to affect its activity. Two different forms of α2,6-ST were shown to be expressed in rat liver cells that differ by a single amino acid (Ma et al., 1997). One possessed a cysteine residue at position 123 (ST-cys) and the other a tyrosine (ST-tyr). ST-tyr had higher activity than ST-cys but it was rapidly cleaved and secreted with half times of three to six hours. ST-cys activity was lower but remained intact in the golgi for up to 24 hours. Disulfide bonded dimers of α2,6-ST have also been found in the golgi of
liver cells and it is believed to form in response to inadequate levels of sugar nucleotide donor or substrates (Ma and Colley, 1996). The enzyme was shown to be catalytically inactive, but it was still able to bind to galactose. Its exact role is unknown, but it may serve as a chaperone molecule, passing acceptor substrates to the catalytically active form of α2,6-ST. It may also serve to prevent the premature exit of asialoglycoproteins from the late golgi. It is not known at present whether any of these post-transcriptional modifications are occurring in uterine epithelial cells.

Other glycosyltransferases whose levels are regulated in the uterus during early pregnancy have been reported. Galactosyltransferase (GT) is the enzyme responsible for the terminal addition of D-Gal. The activity of this enzyme was found to increase eight-fold during the peri-implantation period in mice (Chavez, 1986). Together with the decrease in terminal α2,6-linked sialic acids, these findings explain the increase in terminal galactose seen in the glycocalyx of uterine epithelial cells at this time (Chavez and Anderson, 1985). The activity of α-1,2-Fucosyltransferase (α1,2-FT), the enzyme responsible for the terminal addition of fucose was examined in the luminal epithelium of mice during early pregnancy (White and Kimber, 1994). Enzyme activity was high on days one and two of pregnancy, but was undetectable by day four. Activity levels of α-1,2-FT correlate well with the expression of LNF-1 structures seen in mouse uterine epithelial cells during early pregnancy (Kimber, 1994).
Other Factors

A number of other factors may contribute to the decrease in the levels of α2,6-linked sialic acids. One possibility could be that the donor substrate, CMP-NeuAc, or the acceptor substrate for α2,6-ST is limiting. Most terminal glycosyltransferases compete for common acceptor substrates (Paulson and Colley, 1989). For example, α2,6-ST and α2,3-Sialyltransferase both compete for the Galβ1-4GlcNAc sequence on terminal branches of N-linked oligosaccharides. If the substrate is limiting, one can assume that the type of sialic acid linkage produced will depend on the relative abundance of the two enzymes in the cell.

The presence of α2,6-linked sialic acids may also be controlled by sialidases and proteases. Murine tissues contain four different types of sialidase differing in location and enzymatic properties. They are located mainly in the lysozomal matrix but have also been found in the cytosol and plasma membrane (Kopitz et al., 1996). Proteases have been found in the uterine luminal fluid that are responsible for digesting the extracellular portion of membrane bound glycoconjugates (Finlay et al., 1983; Negishi et al., 1998).

Another possible explanation for changes in α2,6-sialoglycans is that they are actively endocytosed and degraded in lysozomes. Active endocytosis of luminal fluid and plasma membrane components occurs just prior to and during the adhesion stage of implantation in mice (Parr and Parr, 1977). An increase in lysozomal activity also occurs during this period (Kirk and Murphy, 1990). Changes in the
expression of heparan sulfate proteoglycans has been reported by Morris et al. (1988). Similarly, Potter et al. (1996) have reported changes in E-cadherin in luminal epithelial cells. However, neither molecules demonstrated to be located on the apical membrane.

**ROLE OF ESTROGEN AND PROGESTERONE**

Most of the changes that occur in the uterus during implantation have been correlated with levels of estrogen and progesterone. Expression of α2,6-linked sialic acids in uterine epithelial cells appear to also be under the control of the steroid hormones. Levels of expression of α2,6-linked sialic acids during the estrous cycle were shown in this study to be stage dependent. Highest expression was found during estrus and metestrus, soon after estrogen levels are at their peak in the uterus. No expression was observed during diestrus, which coincides with the period when estrogen levels are low and when progesterone levels dominate in the uterus (Fig. 2 and 22). In uteri of ovariectomized mice, low levels of α2,6-linked sialic acids were present in the controls, which probably represents basal levels of expression in the absence of hormonal influence. Mice injected with estradiol showed an increase in the levels of α2,6-linked sialic acids, while no expression was detected in mice injected with progesterone. These results suggest that estradiol stimulates and progesterone inhibits the expression of α2,6-linked sialic acids.
Results of these experiments demonstrate that hormonal regulation of the expression of α2,6-linked sialic acids correlates with the regulation of α2,6-ST. Expression of α2,6-linked sialic acids appear whenever ST mRNA and the protein are present (Fig. 22). It is generally believed that the expression of specific glycosyltransferases in a particular cell will determine the types of oligosaccharide structures made by that cell (Kitagawa and Paulson, 1994; Wang et al., 1990). The pattern of α2,6-linked sialic acid expression in uterine epithelial cells supports this.

Estrogen interacts with estrogen receptors (ER) forming an activated Estrogen-ER complex. This complex binds to cognate DNA sequences that interact with transcriptional regulatory factors to stimulate gene expression (Carson-Jurica et al., 1990). Exactly how estrogen is stimulating levels of α2,6-ST at the transcriptional level in uterine epithelial cells is not known. In hepatic cells, activation of α2,6-ST is through the glucocorticoid receptor pathway (Wang et al., 1990). Dexamethasone was shown to stimulate transcription while RU486, a glucocorticoid antagonist (also an anti-progestin), decreased transcription. Stimulation by dexamethasone enhances transcription of α2,6-ST, using the same initiation site as for basal level expression. The location and nature of cis acting regulatory elements of α2,6-ST has not been determined (Wang et al., 1990).

Because α2,6-ST is expressed in most tissues (Kaneko et al., 1995), differential regulation from a single gene is through different transcriptional initiation points and promoter regions. This may create different isoforms of α2,6-ST
heterogeneously sized from one gene expressed in different tissues. Different isoforms have been identified in human B lymphoblastoid cells (Wang et al., 1993) rat hepatic cells (Ma et al., 1997), rat kidney cells (O'Hanlon and Lau, 1992), and in human placenta (Grundmann et al., 1990).

Tissue differences in expression are achieved in a different manner by α1,3-fucosyltransferase. In contrast to α2,6-ST in which a single gene is regulated by multiple promoter regions, multiple fucosyltransferase genes encoding catalytically similar enzymes are differentially expressed in different tissues (Weston et al., 1992).

Activity levels of sialyltransferase enzymes have been reported to be affected by estrogen and progesterone. Total sialyltransferase activity was examined in rat uterine endothelium during the estrous cycle and in ovariectomized rats given hormone replacement (Nelson et al., 1977; Nelson et al., 1975). Results showed that estradiol stimulates and progesterone inhibits the activity of sialyltransferases in the uterus. The availability of acceptor and donor substrates for α2,6-ST was concluded to be under the control of the steroid hormones. Rat uterine sialic acid concentration was found directly proportional to the level of estrogen and inversely proportional to the level of progesterone. In fact, the level of sialic acid rose during early pregnancy soon after the surge of estrogen (Coppola and Ball, 1966). The concentration of estrogen during the surge was low compared to proestrous estrogen (McCormack and Greenwald, 1974) which may explain why levels of α2,6-ST was unaffected during this period. N-glycosylation is stimulated in mouse uteri by estrogen (Carson et al.,
1990), especially N-linked lactosaminoglycans (Dutt et al., 1986), so it can be assumed that more donor substrates for α2,6-ST are available when estrogen levels are high.
Summary diagram comparing levels of \( \alpha 2,6 \)-ST mRNA, protein and \( \alpha 2,6 \)-linked sialglycoconjugates. For ease of comparison, a value between 0 and 4 were assigned to indicate the intensity of staining (0 = lowest level of staining, 4 = highest). The timing and levels of expression between \( \alpha 2,6 \)-ST mRNA, protein and \( \alpha 2,6 \)-linked sialglycoconjugates is in good correlation during the estrous cycle and early pregnancy. This is probably due to its regulation by the steroid hormones. Comparison with the pattern of hormonal secretion suggests that \( \alpha 2,6 \)-linked sialic acid is stimulated by estrogen and inhibited by progesterone. This is also confirmed in the results seen with ovariectomized mice given hormone replacement.
ROLE FOR A DECREASE IN LEVELS OF $\alpha$2,6-
SIALOGLYCANS DURING IMPLANTATION

What role could a decrease in $\alpha$2,6-linked sialoglycoconjugates have in the process of blastocyst adhesion? It is known that sialic acids can have a negative affect on cellular interactions (Schauer, 1982; Varki, 1997). Due to its negative charge, sialic acids can interfere with cellular interactions by charge repulsion (Morris and Potter, 1984).

Terminal sialic acids have also been found to mask subterminal oligosaccharide structures that may be involved with adhesion. The lectin activity of CD22 is restricted by endogenous sialylation in resting B-cells. Unmasking occurs transiently to modulate interactions during B-cell activation (Razi and Varki, 1998). Serum glycoproteins are protected from clearance by the masking of sialic acids (Drickamer, 1991). A similar situation may be occurring in the uterus. $\alpha$2,6-linked sialic acids may be masking potential molecules involved in the adhesion of the blastocyst.

Large glycoproteins that contain $\alpha$2,6-linked sialic acid could also serve as inhibitory molecules that block adhesion by creating a physical barrier. A model for blastocyst attachment implicating Muc-1 as an inhibitory molecule was proposed by Carson et al. (1998). In this model, they suggested that Muc-1 acts as a barrier during the non-receptive state of the uterus due to its large size. Loss of Muc-1
during conversion to the receptive state allows the apical surface of the uterine epithelium to become accessible to the embryo. They also suggested that heparan sulfate interacting proteins, HB-EGF and amphiregulin were involved in the attachment process.

This model works well for mice where Muc-1 expression decreases at the period of implantation (Braga and Gendler, 1993). In humans, the level of Muc-1 remains unchanged (Hey et al., 1994). What does change in humans is the complexity of carbohydrates attached to Muc-1, creating different glycoforms of the glycoprotein. One of the major Muc-1 glycoforms found during the receptive period lacks sialic acids (DeLoia et al., 1998).

A possible model for blastocyst attachment involving sialic acids is presented here (Fig. 23). There is a decrease in the levels of α2,6-sialylglycans at the time of implantation. Possible explanations for this decrease: 1) removal of sialic acid to unmask potential binding sites, 2) to reduce negative charge repulsion between embryo and uterine epithelium, and 3) removal of large inhibiting glycoproteins that promote a physical barrier. This would then allow potential receptor-ligand, sugar-lectin, or any other type of interactions to occur.

**CONCLUSION**

This study showed a decreased α2,6-linked sialic acid expression on the surface of uterine epithelial cells at the time of implantation. This decrease was
correlated with a decrease in the levels of α2,6-ST present in the cell during this period. Like many other changes that occur in the uterus, the levels of α2,6-ST and sialoglycoconjugates were shown to be under the control of the steroid hormones. A possible role for the decrease in α2,6-linked sialic acids was suggested, but additional experiments are required to confirm this mechanism.

Very little is known about sialyltransferases and its regulation in the expression of sialoglycans in the uterus. Molecular and biochemical studies in other systems have been informative. Molecular studies of uterine endometrium have been impeded in the past by the lack of a cell culture system that retains the hormonally induced differentiation occurring in vivo. Methods for culturing and maintaining polarized epithelial cells in vitro are now possible (Julian et al., 1992; Pimental et al., 1996). With this system, experiments can be performed to characterize the effects of α2,6-linked sialic acids on blastocyst adhesion.
Figure 23: Schematic diagram of a possible model for the initial stage of implantation.

A. Receptive

B. Ligand/receptor masked by sialic acid

Modification of a model proposed by Carson et al. (1998). During the non-receptive period (A), sialic acids block embryo attachment. They may mask adhesion ligands, or may be part of large glycoproteins (Muc-1) that interfere with cell interactions. Loss of sialic acids and sialoglycoproteins during the receptive period (B) permits complementary adhesion molecules on the embryo (i.e. HSPG, EGFR) and uterine epithelial cell surface (i.e. HB-EGF, and glycoproteins containing the LNF-1 epitope) to interact, allowing blastocyst attachment to occur.
Differential expression of sialic acid linkage types have been reported to take place during differentiation, development and in diseased states. The expression of α2,3 and α2,6-linked sialic acids were examined in decidualizing uterine stromal cells in the pregnant rat and mouse (Jones et al., 1996). α2,6-linked sialic acid levels decreased, while α2,3-linked sialic acid increased during stromal cell differentiation. In developing thymocytes, α2,6-sialoglycoconjugates were expressed only in mature thymocytes (Baum et al., 1996). There was also an increased level of α2,6-linked sialic acid over α2,3-linked sialic acid in human colon carcinomas. It would be very informative to examine relative levels of α2,3 and α2,8-linked sialic acids and the sialyltransferases that synthesize their linkage in uterine epithelial cells. Differential regulation of different sialic acid linkage types may be occurring to help create a receptive uterus for blastocyst implantation.

Some insight into sialyltransferases and their regulation in the uterus may be gained from studies done on tumor invasion and metastases. Strickland and Richards (1992) and Denker (1993) brought to our attention how closely related these two processes are to one another. The process of implantation starts from a single cell, the egg. After fertilization, it travels toward the uterus where it implants itself and begins to invade its surrounding tissue. A similar series of events occurs with tumor cells. To metastasize, a tumor cell must escape from its tissue of origin, travel through the blood stream to reach a secondary tissue in which to invade.
Considering these similarities, it is likely that similar mechanisms are involved in both processes. It has already been recognized that both trophoblast cells and tumor cells express similar matrix receptors and are capable of invading its surrounding tissue through the use of similar proteases (Testa and Quigley, 1990). There has been accumulating evidence of sialyltransferases and its involvement in tumor development and metastasis (Dall'Olio et al., 1993; Fukuda, 1996; Sata et al., 1991). Some of this information may be applied to learning more about sialyltransferases in the uterus.

This study was successful in showing changes in the levels of α2,6-linked sialic acid glycoconjugate on the surface of uterine epithelium. It also showed that these changes correlate well with changes in the expression levels of α2,6-ST and its mRNA. Further research in this area is necessary to understand the role that sialic acids play during implantation. The identification of the molecules that contain α2,6-linked sialic acid and their effect on implantation would be very informative in understanding the mechanisms involved in the early stages of blastocyst implantation.
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