AN ABSTRACT OF THE THESIS OF

Georgeen S. Gaza-Bulseco for the degree of Master of Science in Zoology presented on June 1, 2000.

Title: Steroids Regulate α2,6-Sialic Acid-Containing Glycoconjugates in

Murine Uterine Epithelium at the Time of Implantation

Redacted for Privacy

Abstract approved:_____

John E. Morris

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

by

Georgeen S. Gaza-Bulseco

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented June 1, 2000
Commencement June 2001

Master of Science thesis of <u>Georgeen S. Gaza-Bulseco</u> presented June 1, 2000

APPROVED:

Redacted for Privacy

_____

Major Professor, representing Zoology

Redacted for Privacy

_____

Chair of Department of Zoology

Redacted for Privacy

_____

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Redacted for Privacy

Georgeen S. Gaza-Bulseco, Author

ACKNOWLEDGMENTS

TABLE OF CONTENTS

TABLE OF CONTENTS, CONTINUED

LIST OF FIGURES

LIST OF FIGURES, CONTINUED

STEROIDS REGULATE α2,6-SIALIC ACID-CONTAINING GLYCOCONJUGATES IN MURINE UTERINE EPITHELIUM AT THE TIME OF IMPLANTATION

CHAPTER 1

INTRODUCTION

OVERVIEW

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 oligosaccharide 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 sialyl-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 $\alpha 2,6$-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. Influenza

viruses isolated from human bind to the NeuAcα2,6-Gal sequence (Rogers and

Paulson, 1983). The lymphocyte B-cell surface receptor CD22β binds specifically to

oligosaccharides containing α2,6-linked sialic acids (Kelm 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. β-Galactoside α2,6-Sialyltransferase (α2,6-ST) is the enzyme that mediates

the transfer of sialic acid to exposed Galβ1-4GlcNAc 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 attchment, 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.
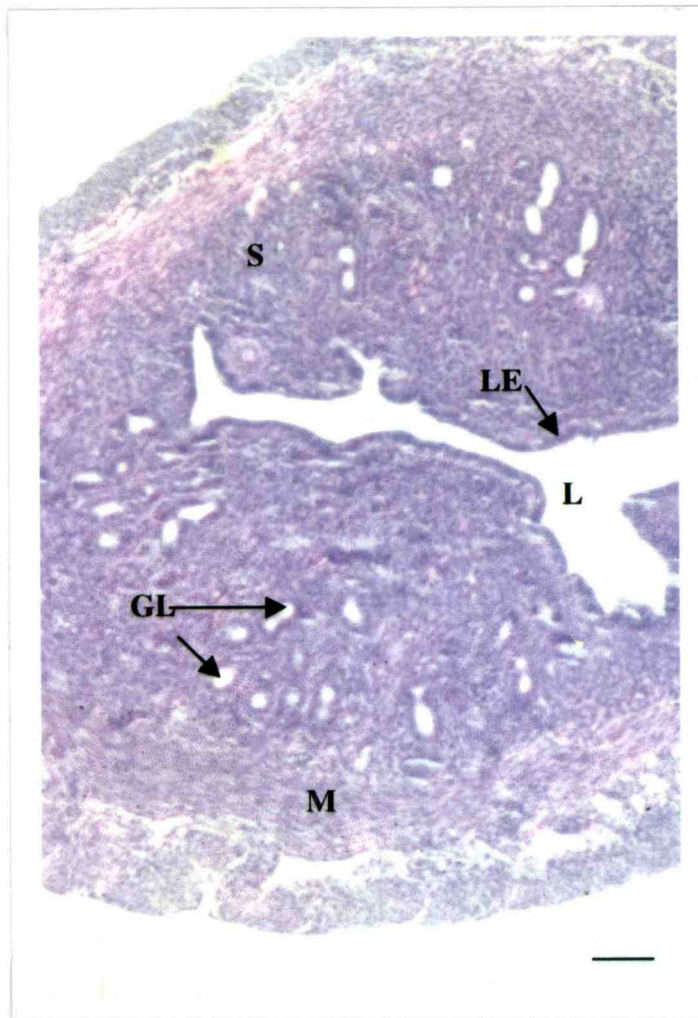
Paraformaldehyde fixed cross section of a mouse uterus in proestrus stained with haematoxylin and eosin to demonstrate cellular components. Luminal epithelial cells (LE); stroma (S); myometrium (M); uterine glands (GL); and lumen (L). Bar = 100uM.

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 recptivity 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.
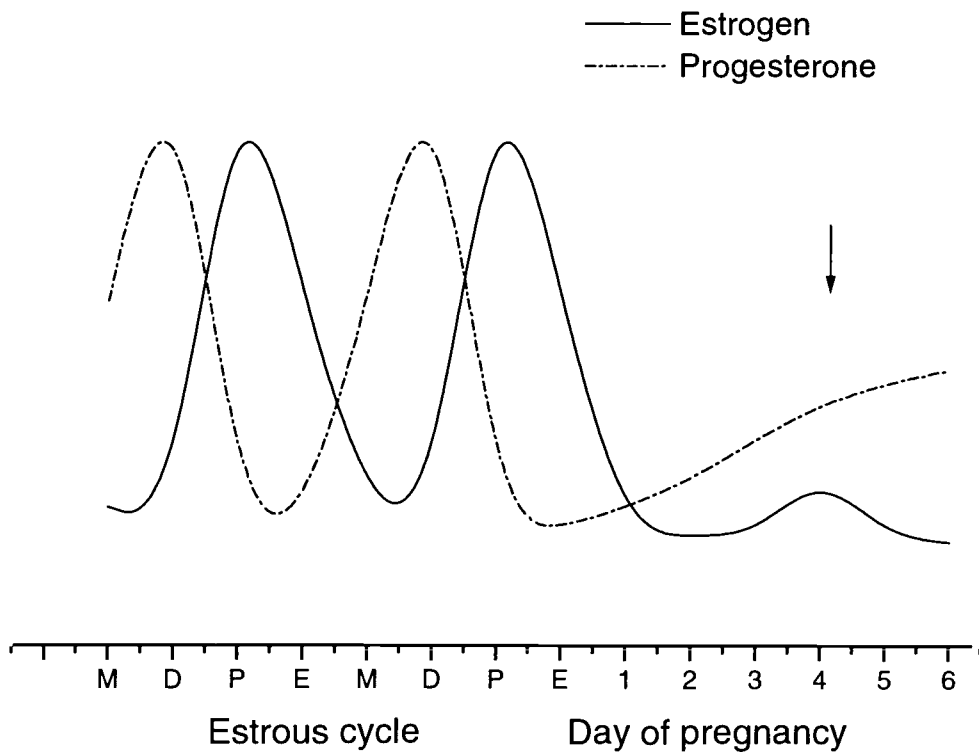
Schematic diagram showing the timing of estrogen and progesterone fluctuations in mice during the estrous cycle and early pregnancy (constructed after McCormack and Greenwald, 1974 and Walmer et. al, 1992). Units shown are arbitrary and do not reflect the relative levels of estrogen and progesterone. 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 communicus agglutinin (RCA-1) 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-1 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

molecule. This is consistent with the anti-adhesive role of mucins in other systems (Hilkens et al., 1995; Kemperman et al., 1994; Ligtenberg et al., 1992).

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-hydoxyl group of N-acetylglucosamine (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.

Figure 3: Structure of sialic acid.
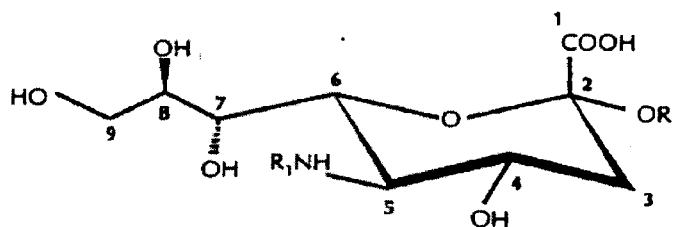
The most common form of sialic acid is N-Acetylneuraminic acid, where R_1 = acetyl. N-Glycolylneuraminic acid is another form of sialic acid, where R_1 = glycolyl. Numerous other forms of sialic acid is made possible with different O- and N-substituents on carbons 4, 7, 8 and 9 (Schauer, 1982).

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 $\alpha 2,3$ linkage, Neu5Acα2,3Galβ1-4(Fucα1-3)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 $\alpha2,3$-linked sialic acids (Crocker et al.,

1994). CD22 is a B-cell specific molecule which binds to ligands on B-lymphocytes

that contain $\alpha2,6$-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 $\alpha2,3$-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 hydoxyl 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 NH_2-

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                                                          TrpGl
   501                                                          TGGCA
                                                                ACCGT


    +3  nArgCysAla ValValSerSer AlaGlySer LeuLysAsn SerGlnLeuG
   551  AAGGTGTGCC GTCGTCTCTT CTGCAGGATC TCTGAAAAAC TCCCAGCTTG
        TTCCACACGG CAGCAGAGAA GACGTCCTAG AGACTTTTTG AGGGTCGAAC


    +3  lyArgGluIle AspAsnHis AspAlaValLeu ArgPheAsn GlyAlaPro
   601  GTCGAGAGAT TGATAATCAT GATGCAGTTC TGAGGTTTAA TGGGGCCCCT
        CAGCTCTCTA ACTATTAGTA CTACGTCAAG ACTCCAAATT ACCCCGGGGA


    +3  ThrAspAsnPhe GlnGlnAsp ValGlySer LysThrThr
   651  ACCGACAACT TCCAACAGGA TGTGGGCTCA AAAACTACC
        TGGCTGTTGA AGGTTGTCCT ACACCCGAGT 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 seqence 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 $\alpha 2,6$-ST in uterine epithelial cells. Following the expression levels of $\alpha 2,6$-ST in uterine epithelial cells during peri-implantation will provide insights into the control of $\alpha 2,6$-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 $\alpha 2,6$-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 $\alpha 2,6$-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 $\alpha 2,6$-ST, the enzyme responsible for synthesis of sialic acids in $\alpha 2,6$-

glycosidic linkages, was followed through these stages.

Results show that the levels of $\alpha 2,6$-linked sialoglycoconjugates decreases at

the time of implantation. This decrease correlates with the decrease in expression of

the enzyme, $\alpha 2,6$-ST. Levels of $\alpha 2,6$-ST and $\alpha 2,6$-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 Boerhinger 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 (E_2) or 100

µg/mouse progesterone (P_4) 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.

Figure 5: Appearance of vaginal smears showing the stages of the estrous cycle.
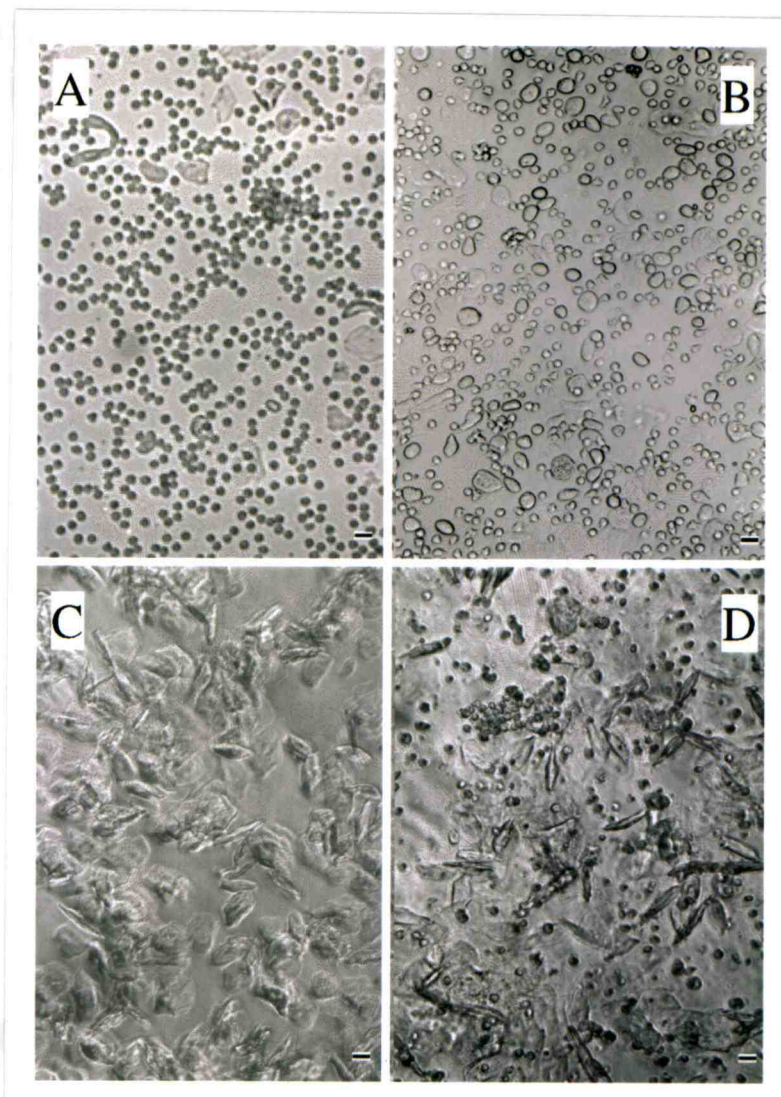
Stages of the estrous cycle were determined by the type and appearance of cells in vaginal smears. (A) diestrus: limited mostly to leukocytes; (B) proestrus: equal number of leukocytes and small, round, nucleated epithelial cells; (C) estrus: large, flattened anucleated epithelial cells with irregular and folded edges; and (D) metestrus: equal numbers of leukocytes and epithelial cells of the morphology seen during the estrus stage. Bar = 10uM.

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 phenylmethylsulfonylfluoride, 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 μg/ml streptavidin-HRP in TBST/Ca^{2+} for one hour at room temperature. After

rinsing with TBST/Ca^{2}, protein bands containing α2,6-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 μm 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 μg/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

μg/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 α2,6-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/Ca^{2+} 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 acetylating positive charges on molecules in the tissue, thereby reducing non-specific interactions with the negative RNA probe.

Figure 6: Diagram of pBluescript II K/S+ with α2,6-sialyltransferase inserted at the EcoRI site in the open reading frame.
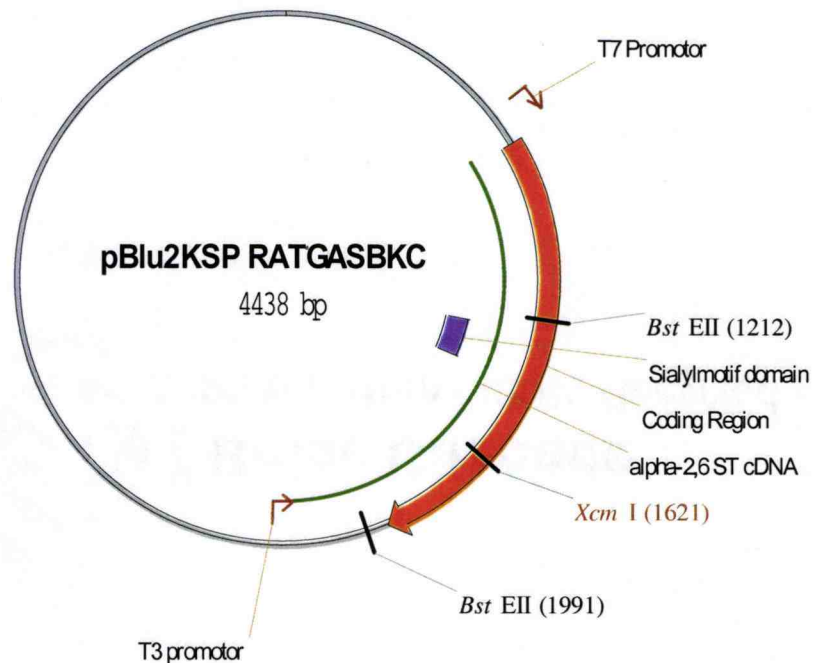
The alpha 2,6-ST cDNA (shown in green) was inserted in the EcoR1 site of pBluescript II K/S+. The coding region for alpha 2,6-ST is shown in red. The sialylmotif domain is shown in purple. Digoxigenin labeled probes were prepared with digoxigenin-UTP and the appropriate RNA polymerase. Antisense probes 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 $MgCl_2$). Hybridization was

detected by covering the sections with detection buffer containing 0.18mg/ml nitro

blue tetrazolium (NBT), 0.34 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP),

and 240 ug/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 H_2O and mounted with glycerol.

CHAPTER 3

RESULTS

LEVELS OF α2,6-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 α2,6-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 α2,6-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 uM.

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.

Figure 8: Changes in α2,6-linked sialic acids in extracts from mice during early pregnancy.
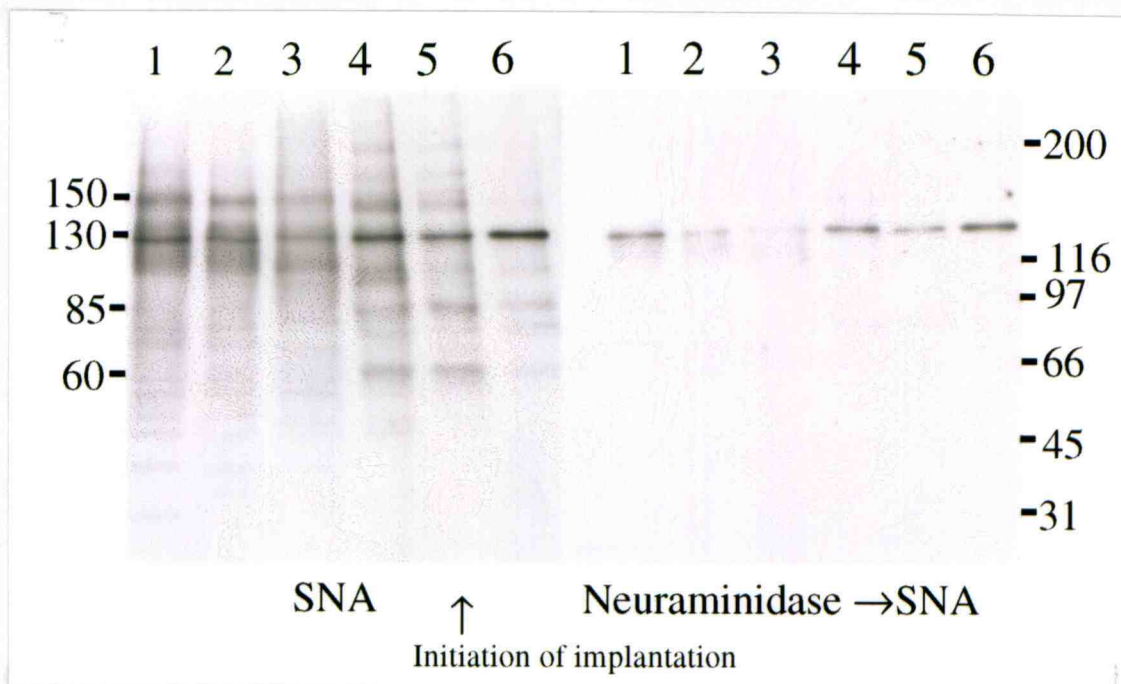
Extracts of uterine luminal epithelial cells from day one through day six pregnant mice were run on SDS-PAGE after adjusting loading for equal protein concentration, and transblotted on to a PVDF membrane. The blot was probed with biotin conjugated SNA followed by avidin-HRP. Bands positive for α2,6-linked sialic acids were detected by chemiluminesence. There was a pronounced change in the profile of SNA-stained bands that occured 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.
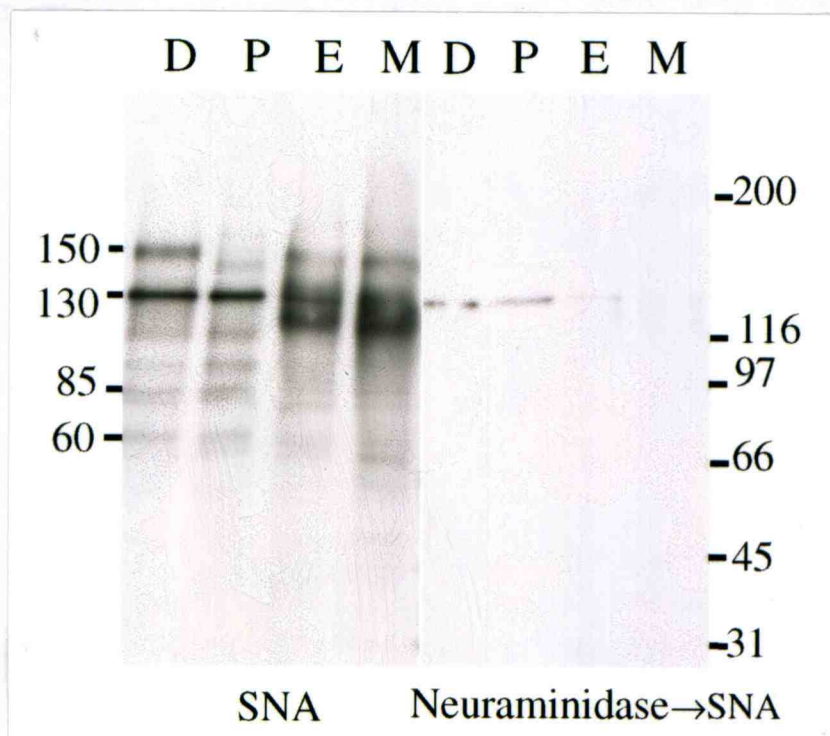
Changes in extracts from mice during the estrous cycle

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.

Figure 9: Changes in α2,6-linked sialic acids in uterine epithelial cell extracts from mice in the different stages of the estrous cycle.

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 chemiluminesence. 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=diestrus, P=proestrus, E=estrus, and M=metestrus.

59

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

$\alpha2,6$-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 $\alpha2,6$-linked sialic acids.

Figure 10: α2,6-linked sialic acid in frozen sections of mouse uterus during early pregnancy.
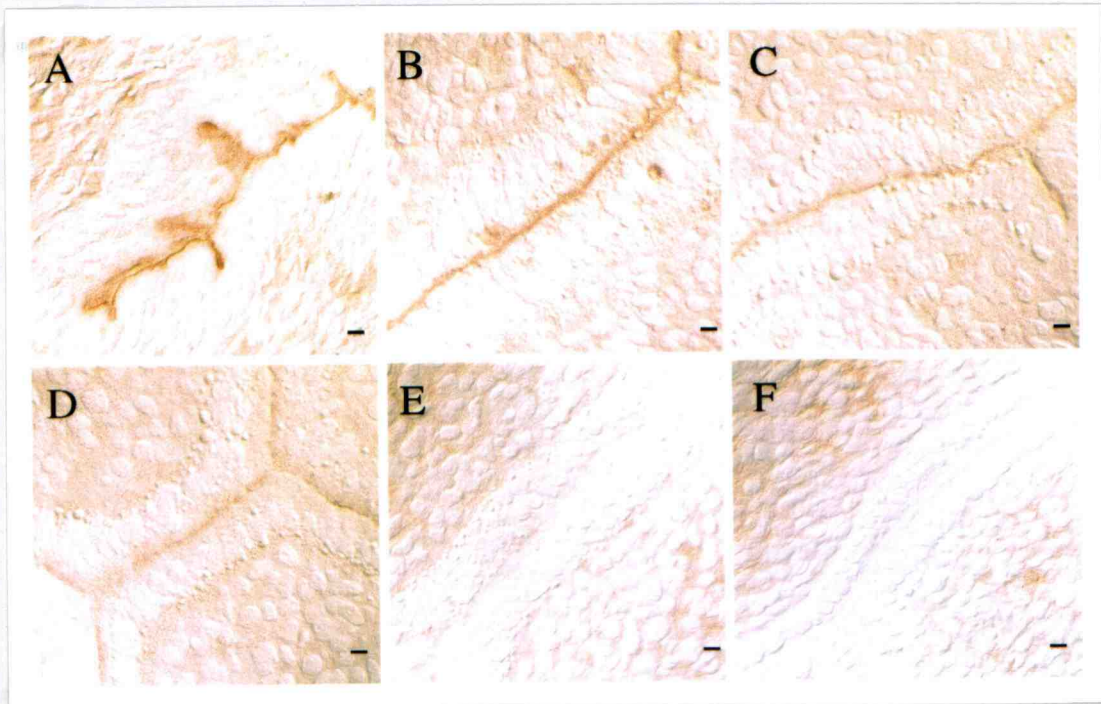
Frozen sections of uteri from day one through day six pregnant mice were probed with biotin-SNA as described in the methods section. Staining was present at the apical surface of the luminal epithelial cells and was most intense on day one of pregnancy (A). Staining decreased gradually through days two (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.
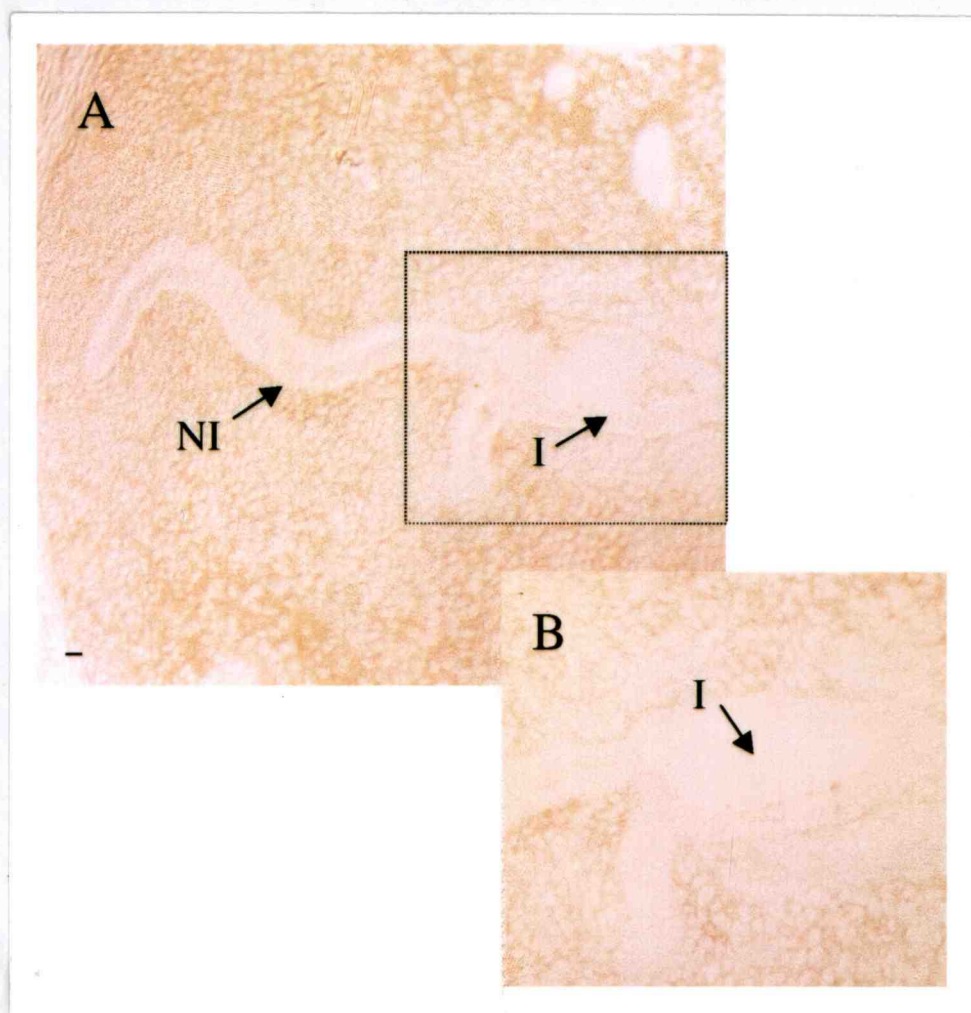
(A) Section through an implantation site from a mouse on day six of pregnancy probed with SNA. No staining was detected at either non-implantation (NI) and implantation (I) sites. Bar = 20 μM. Inset (B) shows the implantation site at a higher magnification.

Figure 12: Frozen sections of mouse uteri during early pregnancy treated with neuraminidase prior to probing with SNA.
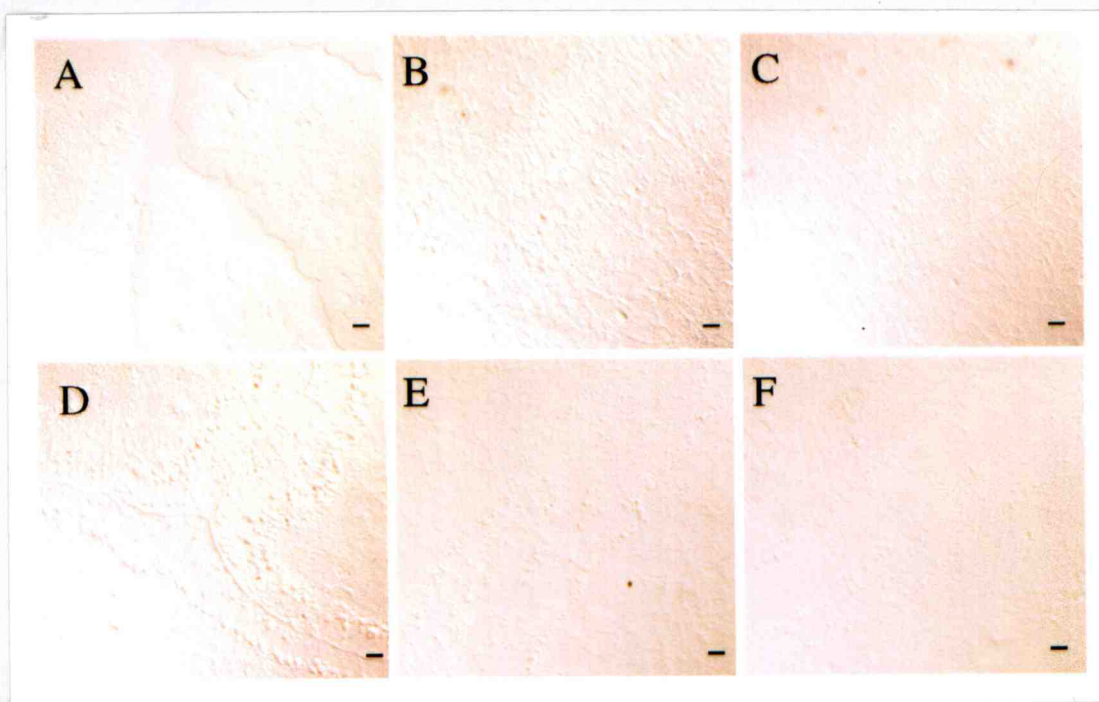
Frozen sections were treated with 1.0 unit neuraminidase for one hour at 37°C prior to probing with biotin-SNA. No staining was detected at the apical surface of uterine epithelial cells on day one through day six of pregnancy. A through F represents day one through day six respectively. Bar = 10 μM.

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).

Figure 13: α2,6-linked sialic acid in frozen sections of mouse uterus during the various stages of the estrous cycle.
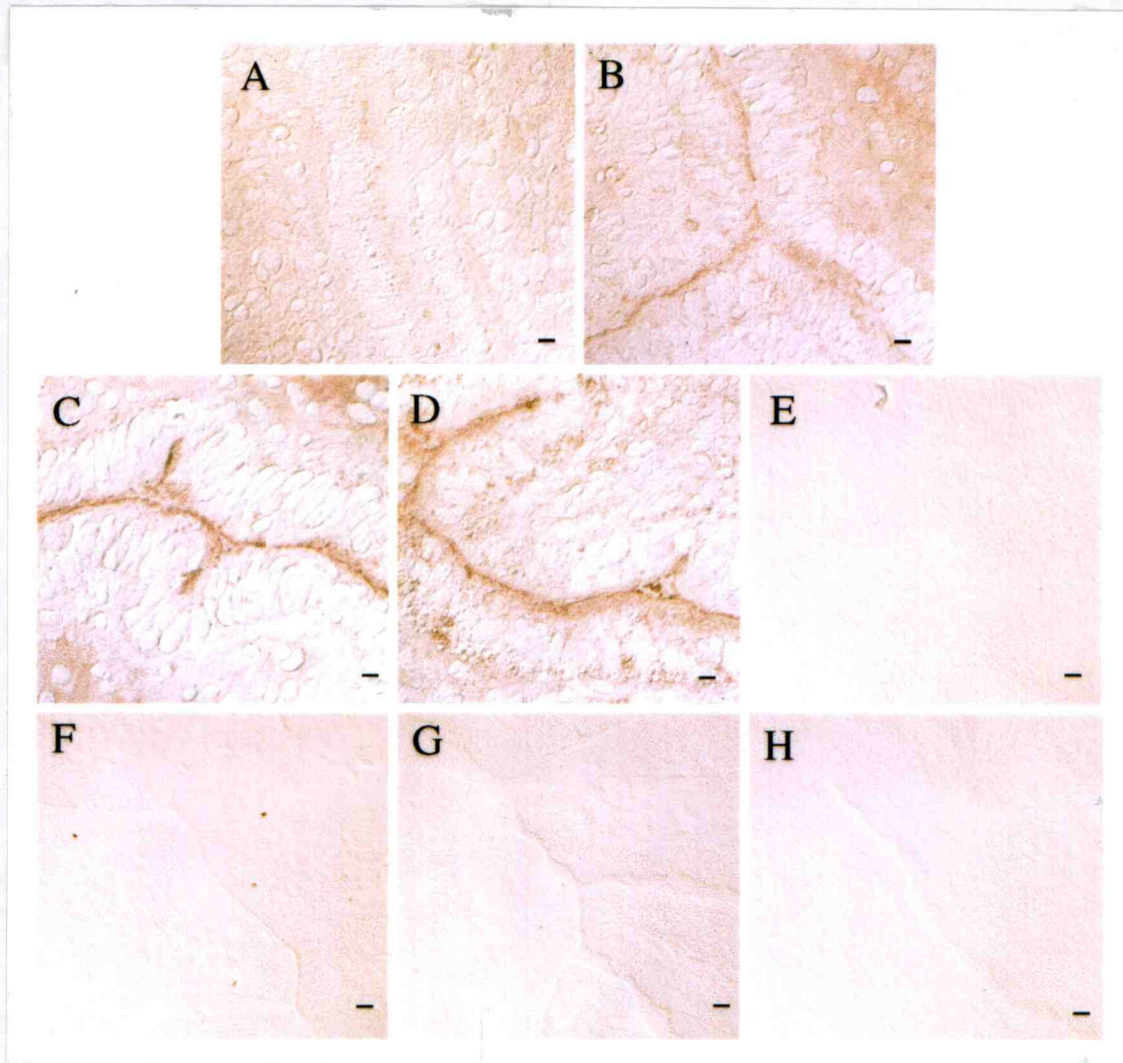
Frozen sections of mouse uteri from various stages of the estrous cycle were prepared and probed with SNA as described in the methods section. A strong signal at the apical surface of the luminal epithelial cells was seen during estrus (C) and metestrus (D). The signal was less intense during proestrus (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.

Figure 14: α2,6-linked sialic acid in sections of uteri from ovariectomized mice.
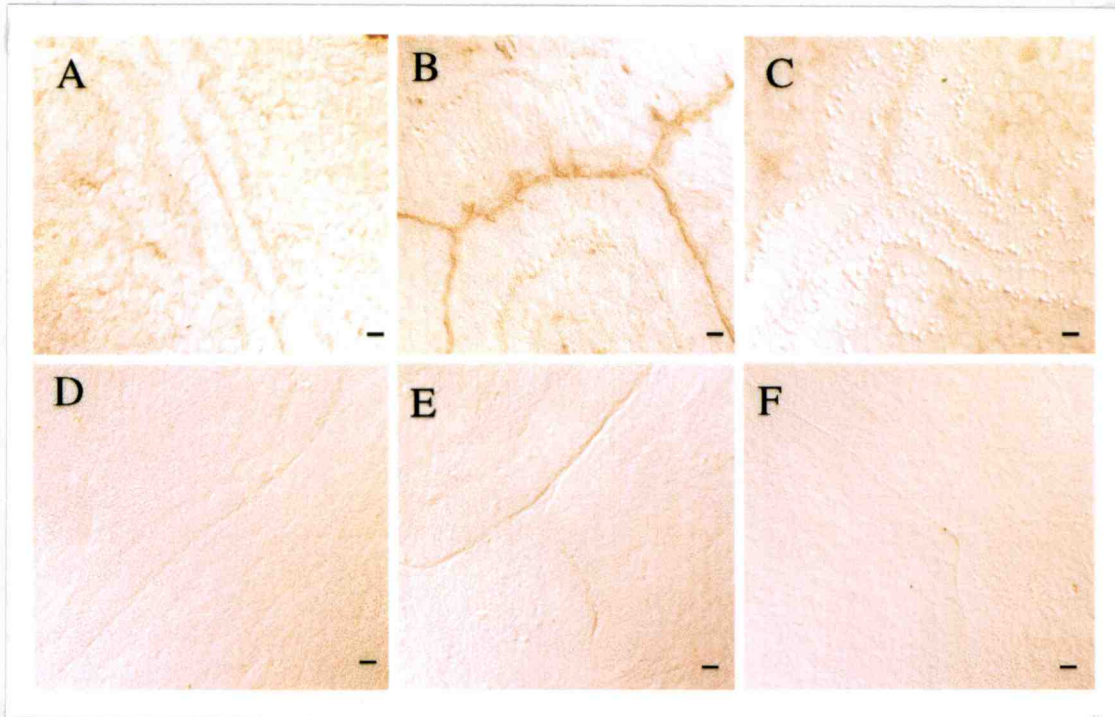
Frozen sections from ovariectomized mice given hormone replacement were probed with biotin-SNA as described in the methods section. Staining was detected only at the apical surface of uterine epithelial cells from mice injected with estradiol alone (B). Less staining was detected in controls given vehicle 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 α2,6- SIALYLTRANSFERASE MRNA IN UTERINE EPITHELIAL CELLS

α2,6-sialyltransferase is the enzyme responsible for the formation of an α2,6-glycosidic linkage between sialic acid and Galβ1-4GlcNAc structures. In situ hybridizations on uterine sections were used to examine the relationship between the levels of α2,6-linked sialic acid and the expression of α2,6-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 α2,6-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 α2,6-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 α2,6-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.
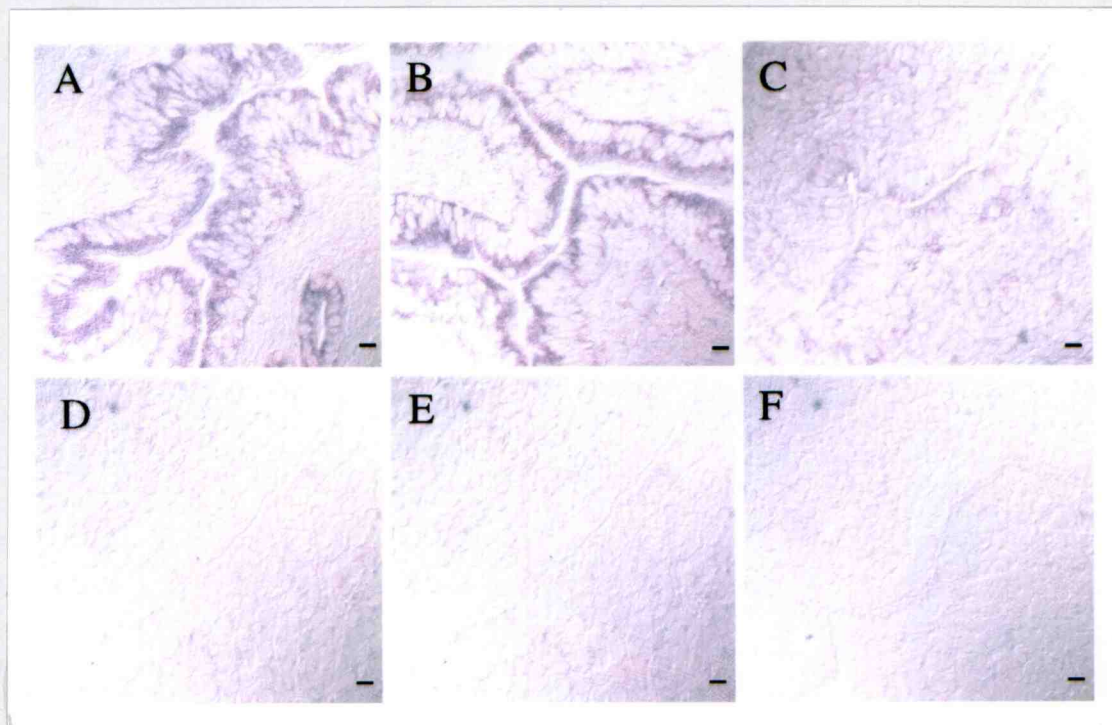
Frozen sections of mouse uteri during early pregnancy were hybridized with an anti-sense digoxigenin labeled α2,6-ST RNA probe as described in the methods section. Staining in the cytoplasm of luminal epithelial cells was most intense on days one (A) and two of pregnancy (B). By day three (C), staining decreased significantly and was not detected on days four (D), five (E), and six (F) of pregnancy. Bar = 10 μM.

Figure 16: In situ hybidization control sections of mouse uteri during early pregnancy
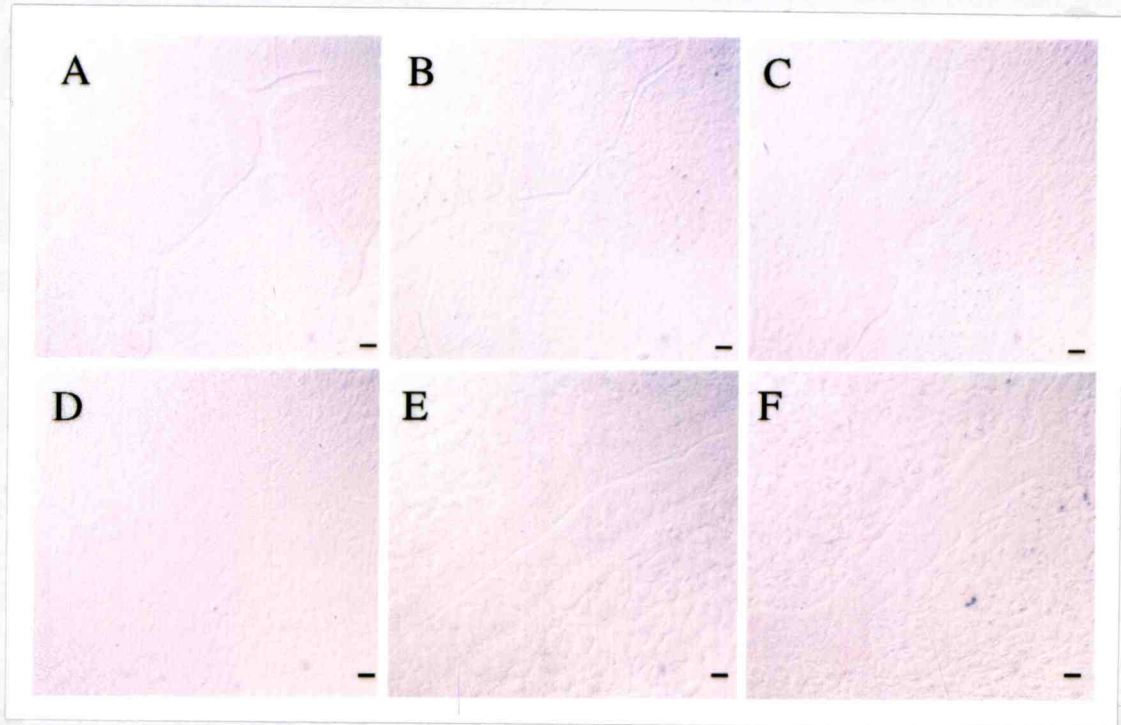
Frozen sections of mouse uteri were hybridized with a sense orientation digoxigenin labeled α2,6-ST RNA probe as described in the methods section. No staining was detected on sections from day one through day six of pregnancy, A through F respectively. Bar = 10 μM.

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.
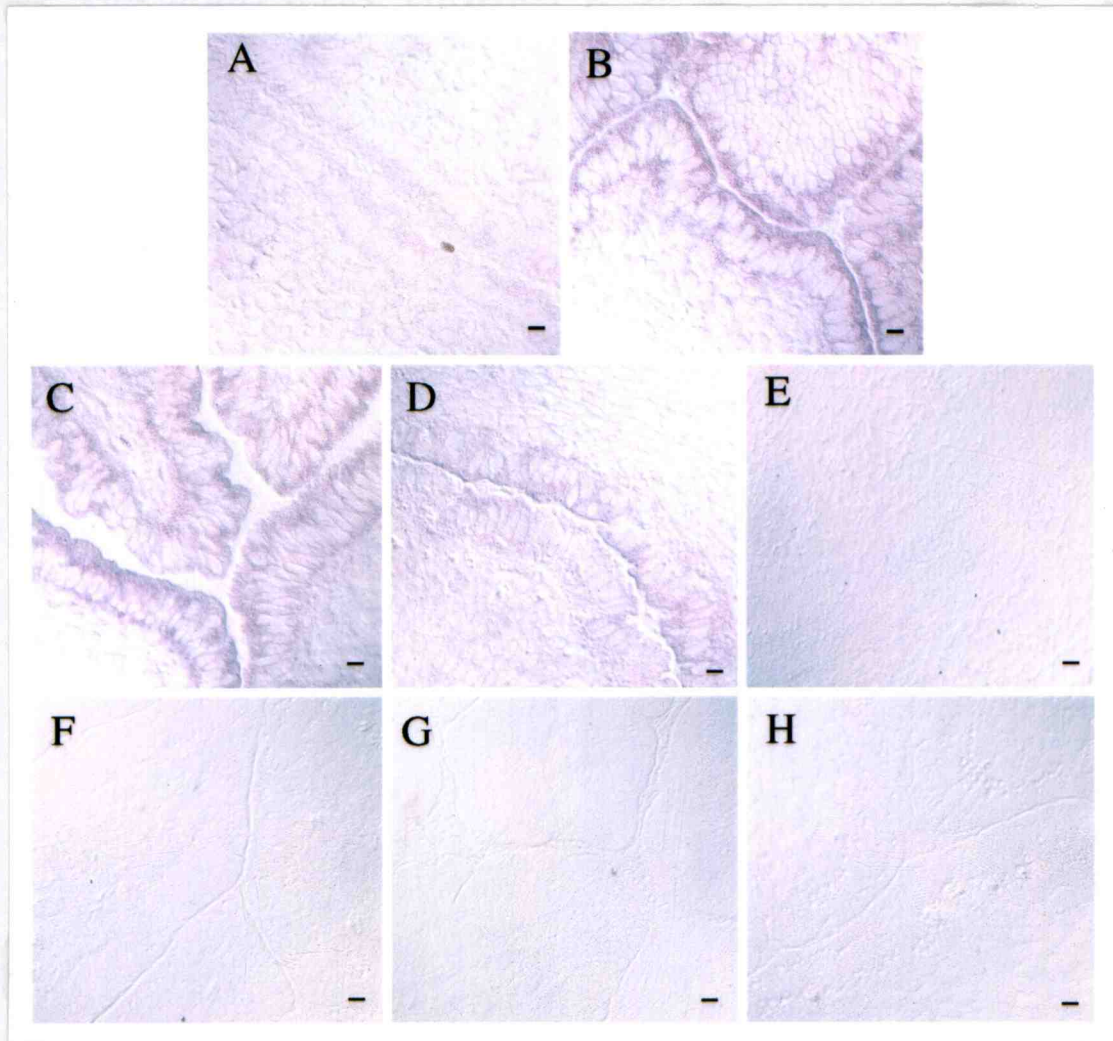
Frozen sections of mouse uteri during the various stages of the estrous cycle were hybridized with an anti-sense digoxigenin labeled α2,6-ST RNA probe as described in the methods section. Staining was most intense in the cytoplasm of uterine epithelial cells during proestrus (B) and estrus (C) and it 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.

Figure 18: Expression of α2,6-ST mRNA in uterine epithelial cells from ovariectomized mice given hormone replacement.
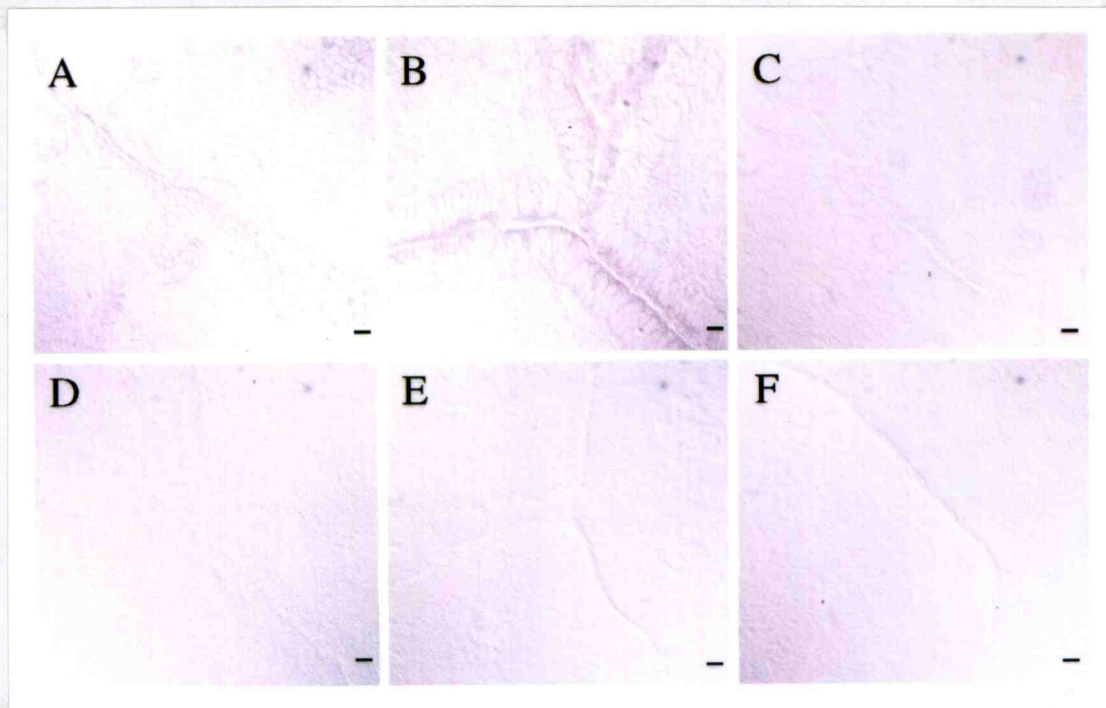
Frozen sections from ovariectomized mice given hormone replacement were hybridized with an anti-sense digoxigenin labeled α2,6-ST RNA probe as described in the methods section. Intense staining was present in uteri from mice injected with estradiol (B). Faint staining was detected in controls given vehicle 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 α2,6-SIALYLTRANSFERASE PROTEIN IN UTERINE EPITHELIUM

Results from this study indicated that the expression pattern of α2,6-ST

mRNA and α2,6-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 α2,6-ST mRNA was highest during

proestrus and estrus, whereas α2,6-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 α2,6-linked sialic acids. In order to have a close

correlation in the timing of the expression of both α2,6-ST mRNA and α2,6-linked

sialic acids, the message must first be translated into a functional protein. To follow

the expression of α2,6-ST protein, sections were probed with a polyclonal antibody

made against α2,6-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 α2,6-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.
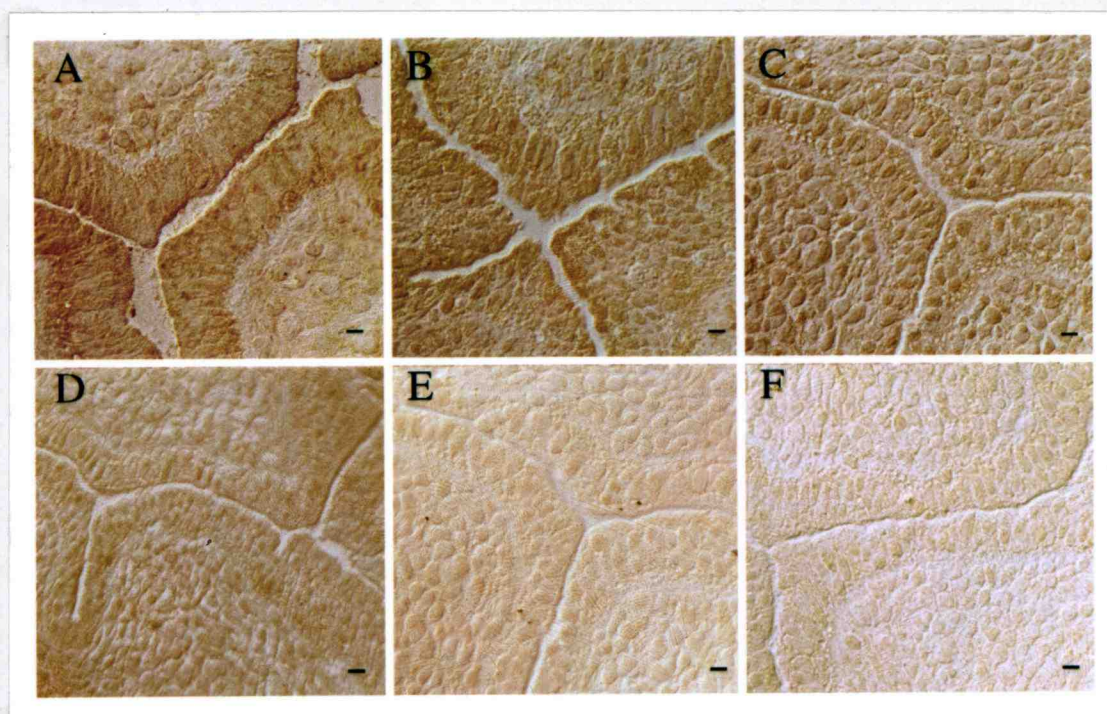
Frozen sections of mouse uteri during early pregnancy were probed with a polyclonal antibody against α2,6-ST as described in the methods section. Staining in the cytoplasm of uterine luminal epithelial cells was most intense during days one (A) and two (B) of pregnancy and it gradually decreased on days 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.
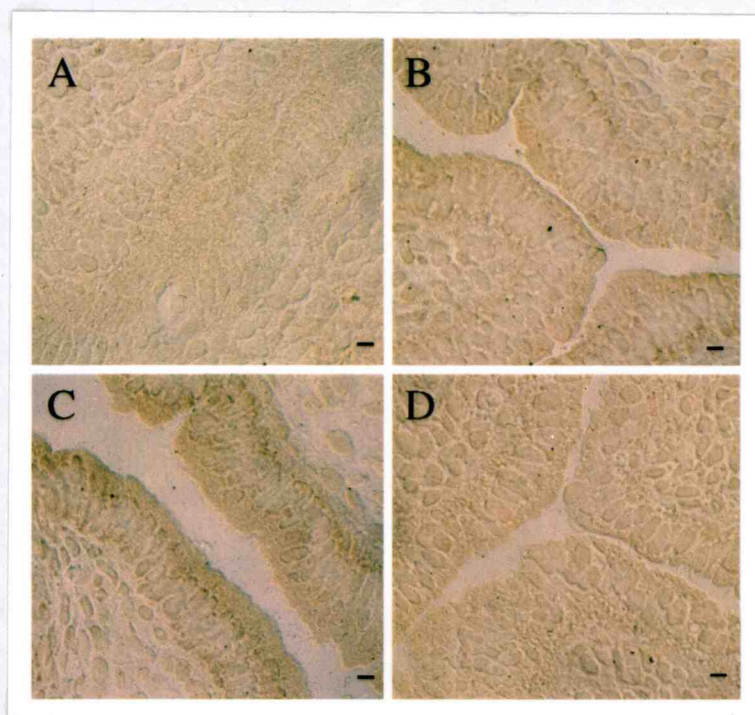
Frozen sections of mouse uteri during the estrous cycle were probed with a polyclonal antibody against α2,6-ST as described in the methods section. Staining was most intense during estrus (C) and less staining was detected during proestrus (B) and metestrus (D). No staining was detected during diestrus (A). Bar = 10 μM.

Figure 21: Expression of α2,6-ST protein in uterine epithelial cells from ovariectomized mice given hormone replacement.
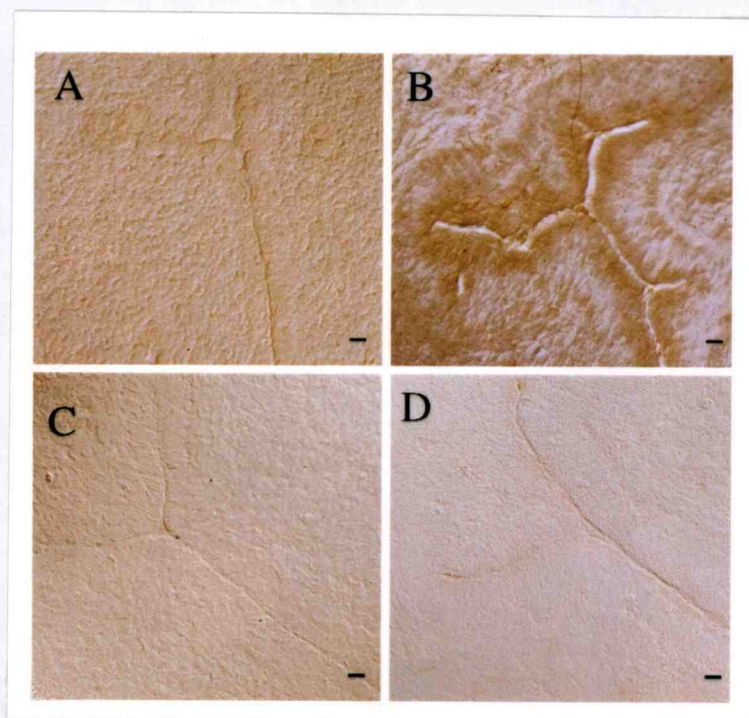
Frozen sections from ovariectomized mice given hormone replacement were probed with a polyclonal antibody against α2,6-ST as described in the methods section. Staining was detected in the cytoplasm of uterine epithelial cells from mice injected with estradiol (B). Faint staining was detected in controls 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.

CHAPTER 4

DISCUSSION

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 $\alpha2,6$-linked sialic acid was clearly observed in uterine sections. The highest levels of $\alpha2,6$-linked sialic acids were found on day one and then gradually decreased up to day four of pregnancy. By SNA staining, no $\alpha2,6$-linked sialic acids were detected on day five at both implantation and non-implantation sites. This suggests that the decrease in $\alpha2,6$-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 $\alpha2,6$-linked sialic acids to have the greatest impact on blastocyst attachment.

This is the first known report showing a decrease in the expression of $\alpha2,6$-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 α2,6-glycosidic linkage to Galβ1-4GlcNAc or to

GalNAcβ1-4GlcNAc (Shibuya et al., 1987). Another glycosidic linkage that is

common in cells is α2,3, 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 α2,3 and α2,6 sialylation in

different cells and tissues. Different levels of α2,3 and α2,6-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 $\alpha 2,6$-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 $\alpha 2,6$-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 $\alpha 2,6$-

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 $\alpha 2,6$-

sialoglycoconjugates being expressed. Last, the increased number of terminal

galactose residues (Chavez and Anderson, 1985) may be due to the lack of $\alpha 2,6$-

linked sialic acids on $Gal\beta 1$-4GlcNAc structures. Taken together, the decrease in

$\alpha 2,6$-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-sialyltranserase

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-sialoglycoconjugates 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-sialogycoconjugates was also high on day one and it gradually decreased up to day four (Fig. 22). From these results, the level of α2,6-sialogycoconjugates 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-sialogycoconjugates 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 $\alpha 2,6$-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 $\alpha 2,6$-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 ($\alpha 1,2$-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 oligosaccaride

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 $\alpha1,3$-

fucosyltransferase. In contrast to $\alpha2,6$-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 $\alpha2,6$-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 $\alpha2,6$-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.

Figure 22: Comparison of expression levels of α2,6-ST mRNA, protein and α2,6-linked sialic acid.
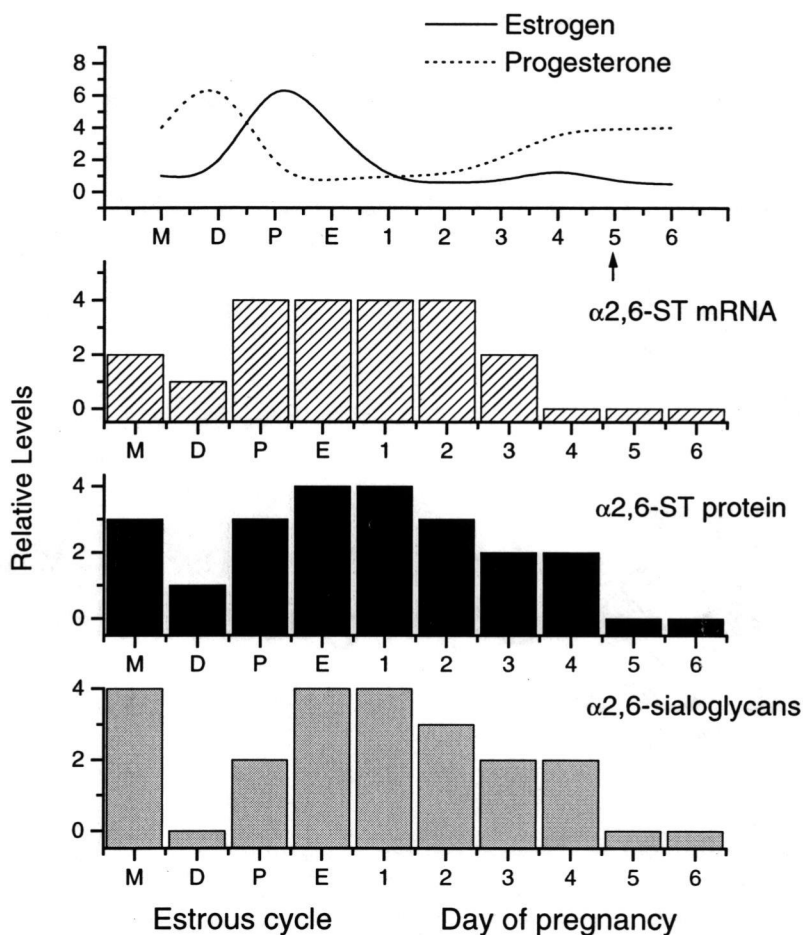
Summary diagram comparing levels of α2,6-ST mRNA, protein and α2,6-linked sialoglycoconjugates. For ease of comparison, a value between 0 and 4 were assigned to indicate the intensity of staining (0 = lowest level of staining, 4 = highest). The timing and levels of expression between α2,6-ST mRNA, protein and α2,6-linked sialoglycoconjugates 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 α2,6-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 α2,6-SIALOGLYCANS DURING IMPLANTATION

What role could a decrease in α2,6-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. α2,6-linked sialic acids may be masking potential molecules involved in the adhesion of the blastocyst.

Large glycoproteins that contain α2,6-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 fournd 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 $\alpha 2,6$-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 $\alpha 2,6$-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.

Blastocyst

B.

Blastocyst

Receptive

e.g.HSPG, EGF

e.g.HB-EGF, LNF1 epitope

Sialic acid residue

Ligand/receptor masked by sialic acid

Large sialic acid containing glycoprotein
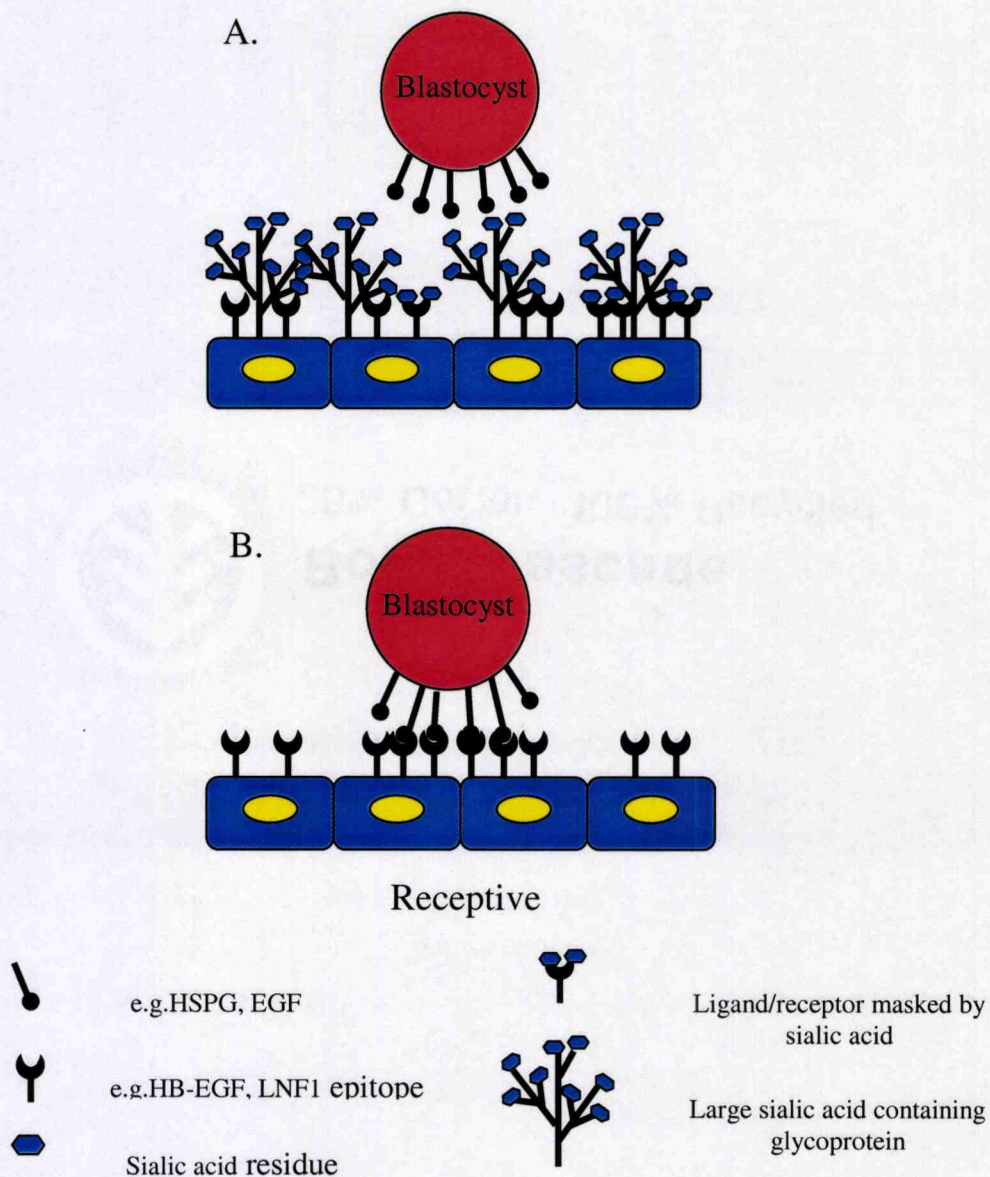
Modification of a model proposed by Carson et al. (1998). During the non-receptive period (A), sialic acids block embryo attachment. They may mask adhesion ligands, or may be part of large glycoproteins (Muc-1) that interfere with cell interactions. Loss of sialic acids and sialoglycoproteins during 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 ghycoproteins 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 $\alpha2,3$ and $\alpha2,6$-linked sialic acids were examined in decidualizing uterine stromal cells in the pregnant rat and mouse (Jones et al., 1996). $\alpha2,6$-linked sialic acid levels decreased, while $\alpha2,3$-linked sialic acid increased during stromal cell differentiation. In developing thymocytes, $\alpha2,6$-sialoglycoconjugates were expressed only in mature thymocytes (Baum et al., 1996). There was also an increased level of $\alpha2,6$-linked sialic acid over $\alpha2,3$-linked sialic acid in human colon carcinomas. It would be very informative to examine relative levels of $\alpha2,3$ and $\alpha2,8$-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 $\alpha2,6$-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 $\alpha2,6$-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 $\alpha2,6$-linked sialic acid and their effect on implantation would be very informative in understanding the mechanisms involved in the early stages of blastocyst implantation.

REFERENCES CITED

Anderson, T. L., and Hoffman, L. H. (1984). Alterations in epithelial glycocalyx of rabbit uteri during early pseudopregnancy and pregnancy, and following ovariectomy. Am J Anat *171*, 321-34.

Anderson, T. L., Olson, G. E., and Hoffman, L. H. (1986). Stage-specific alterations in the apical membrane glycoproteins of endometrial epithelial cells related to implantation in rabbits. Biol Reprod *34*, 701-20.

Baum, L. G., Derbin, K., Perillo, N. L., Wu, T., Pang, M., and Uittenbogaart, C. (1996). Characterization of terminal sialic acid linkages on human thymocytes. Correlation between lectin-binding phenotype and sialyltransferase expression. J Biol Chem *271*, 10793-9.

Berg, E. L., Robinson, M. K., Mansson, O., Butcher, E. C., and Magnani, J. L. (1991). A carbohydrate domain common to both sialyl Le(a) and sialyl Le(X) is recognized by the endothelial cell leukocyte adhesion molecule ELAM-1. J Biol Chem *266*, 14869-72.

Braga, V. M., and Gendler, S. J. (1993). Modulation of Muc-1 mucin expression in the mouse uterus during the estrus cycle, early pregnancy and placentation. J Cell Sci *105*, 397-405.

Bresalier, R. S., Rockwell, R. W., Dahiya, R., Duh, Q. Y., and Kim, Y. S. (1990). Cell surface sialoprotein alterations in metastatic murine colon cancer cell lines selected in an animal model for colon cancer metastasis. Cancer Res *50*, 1299-307.

Carbone, F. R., and Gleeson, P. A. (1997). Carbohydrates and antigen recognition by T cells. Glycobiology *7*, 725-30.

Carson, D. D., DeSouza, M. M., and Regisford, E. G. (1998). Mucin and proteoglycan functions in embryo implantation. Bioessays *20*, 577-83.

Carson, D. D., Farrar, J. D., Laidlaw, J., and Wright, D. A. (1990). Selective activation of the N-glycosylation apparatus in uteri by estrogen. J Biol Chem *265*, 2947-55.

Carson, D. D., Tang, J. P., and Julian, J. (1993). Heparan sulfate proteoglycan (perlecan) expression by mouse embryos during acquisition of attachment competence. Dev Biol *155*, 97-106.

Carson, D. D., Wilson, O. F., and Dutt, A. (1990). Glycoconjugate expression and interactions at the cell surface of mouse uterine epithelial cells and periimplantation-stage embryos. In Trophoblast Invasion and Endometrial Receptivity. Novel Aspects of the Cell Biology of Embryo Implantation, H. W. Denker and J. D. Aplin, eds. (New York: Plenum Medical Book Comp.), pp. 211-241.

Carson-Jurica, M. A., Schrader, W. T., and O'Malley, B. W. (1990). Steroid receptor family: structure and functions. Endocr Rev *11*, 201-20.

Chavez, D. J. (1986). Cell surface of mouse blastocysts at the trophectoderm-uterine interface during the adhesive stage of implantation. Am J Anat *176*, 153-8.

Chavez, D. J., and Anderson, T. L. (1985). The glycocalyx of the mouse uterine luminal epithelium during estrus, early pregnancy, the peri-implantation period, and delayed implantation. I. Acquisition of Ricinus communis I binding sites during pregnancy. Biol Reprod *32*, 1135-42.

Colwell, T. P. (1969). Implantation and development of mouse eggs transferred to the uteri of non-progestational mice. J Reprod Fertil *19*, 239-245.

Coppola, J. A., and Ball, J. L. (1966). Uterine sialic acid in relation to ovarian steroids. Steroids *8*, 345-52.

Couzinet, B., Le Strat, N., Ulmann, A., Baulieu, E. E., and Schaison, G. (1986). Termination of early pregnancy by the progesterone antagonist RU 486 (Mifepristone). N Engl J Med *315*, 1565-70.

Crocker, P. R., Kelm, S., Dubois, C., Martin, B., McWilliam, A. S., Shotton, D. M., Paulson, J. C., and Gordon, S. (1991). Purification and properties of sialoadhesin, a sialic acid-binding receptor of murine tissue macrophages. Embo J *10*, 1661-9.

Crocker, P. R., Mucklow, S., Bouckson, V., McWilliam, A., Willis, A. C., Gordon, S., Milon, G., Kelm, S., and Bradfield, P. (1994). Sialoadhesin, a macrophage sialic acid binding receptor for haemopoietic cells with 17 immunoglobulin-like domains. Embo J *13*, 4490-503.

Dall'Olio, F., Malagolini, N., Guerrini, S., and Serafini-Cessi, F. (1993). Resistance to methotrexate is associated with selective changes of alpha 2,6- and alpha 2,3-sialyltransferase activities toward N- acetyllactosaminic sequences in human colon cancer cell line HT-29. Biochem Biophys Res Commun *196*, 714-20.

Das, S. K., Chakraborty, I., Paria, B. C., Wang, X. N., Plowman, G., and Dey, S. K. (1995). Amphiregulin is an implantation-specific and progesterone-regulated gene in the mouse uterus. Mol Endocrinol *9*, 691-705.

Das, S. K., Chakraborty, I., Wang, J., Dey, S. K., and Hoffman, L. H. (1997). Expression of vascular endothelial growth factor (VEGF) and VEGF- receptor messenger ribonucleic acids in the peri-implantation rabbit uterus. Biol Reprod *56*, 1390-9.

Das, S. K., Wang, X. N., Paria, B. C., Damm, D., Abraham, J. A., Klagsbrun, M., Andrews, G. K., and Dey, S. K. (1994). Heparin-binding EGF-like growth factor gene is induced in the mouse uterus temporally by the blastocyst solely at the site of its apposition: a possible ligand for interaction with blastocyst EGF- receptor in implantation. Development *120*, 1071-83.

Datta, A. K., and Paulson, J. C. (1995). The sialyltransferase "sialylmotif" participates in binding the donor substrate CMP-NeuAc. J Biol Chem *270*, 1497-500.

Debray, H., Decout, D., Strecker, G., Spik, G., and Montreuil, J. (1981). Specificity of twelve lectins towards oligosaccharides and glycopeptides related to N-glycosylproteins. Eur J Biochem *117*, 41-55.

DeLoia, J. A., Krasnow, J. S., Brekosky, J., Babaknia, A., Julian, J., and Carson, D. D. (1998). Regional specialization of the cell membrane-associated, polymorphic mucin (MUC1) in human uterine epithelia. Hum Reprod *13*, 2902-9.

Denker, H. W. (1993). Implantation: a cell biological paradox. J Exp Zool *266*, 541-58.

DeSouza, M. M., Lagow, E., and Carson, D. D. (1998). Mucin functions and expression in mammalian reproductive tract tissues. Biochem Biophys Res Commun *247*, 1-6.

DeSouza, M. M., Mani, S. K., Julian, J., and Carson, D. D. (1998). Reduction of mucin-1 expression during the receptive phase in the rat uterus. Biol Reprod *58*, 1503-7.

Dickmann, Z., and Noyes, R. W. (1961). Fertil Steril *12*, 310-318.

Drickamer, K. (1991). Clearing up glycoprotein hormones. Cell *67*, 1029.

Dutt, A., Tang, J. P., Welply, J. K., and Carson, D. D. (1986). Regulation of N-linked glycoprotein assembly in uteri by steroid hormones. Endocrinology *118*, 661-73.

Enders, A. C., and Nelson, D. M. (1973). Pinocytotic activity of the uterus of the rat. Am J Anat *138*, 277-99.

Enders, A. C., and Schlafke, S. (1969). Cytological aspects of trophoblast-uterine interaction in early implantation. Am J Anat *125*, 1-29.

Enders, A. C., and Schlafke, S. (1967). A morphological analysis of the early implantation stages in the rat. Am. J. Anat. *138*, 277-300.

Enders, A. C., and Schlafke, S. (1974). Surface coats of the mouse blastocyst and uterus during the preimplantation period. Anat Rec *180*, 31-45.

Finlay, T. H., Katz, J., Kadner, S., and Levitz, M. (1983). Properties of an estrogen-induced hydrolytic enzyme from mouse uterus. J Steroid Biochem *19*, 743-9.

Fukuda, M. (1996). Possible roles of tumor-associated carbohydrate antigens. Cancer Res *56*, 2237-44.

Gallagher, J. T., Morris, A., and Dexter, T. M. (1985). Identification of two binding sites for wheat-germ agglutinin on polylactosamine-type oligosaccharides. Biochem J *231*, 115-22.

Gillespie, W., Paulson, J. C., Kelm, S., Pang, M., and Baum, L. G. (1993). Regulation of alpha 2,3-sialyltransferase expression correlates with conversion of peanut agglutinin (PNA)+ to PNA- phenotype in developing thymocytes. J Biol Chem *268*, 3801-4.

Given, R. L., and Enders, A. C. (1981). Mouse uterine glands during the peri-implantation period. II. Autoradiographic studies. Anat Rec *199*, 109-27.

Glasser, S. R., and Mulholland, J. (1993). Receptivity is a polarity dependent special function of hormonally regulated uterine epithelial cells. Microsc Res Tech *25*, 106-20.

Grundmann, U., Nerlich, C., Rein, T., and Zettlmeissl, G. (1990). Complete cDNA sequence encoding human beta-galactoside alpha-2,6- sialyltransferase. Nucleic Acids Res *18*, 667.

Guillomot, M. (1995). Cellular interactions during implantation in domestic ruminants. J Reprod Fertil Suppl *49*, 39-51.

Guillomot, M., Flechon, J. E., and Wintenberger-Torres, S. (1982). Cytochemical studies of uterine and trophoblastic surface coats during blastocyst attachment in the ewe. J Reprod Fertil *65*, 1-8.

Harduin-Lepers, A., Recchi, M. A., and Delannoy, P. (1995). 1994, the year of sialyltransferases. Glycobiology 5, 741-58.

Hewitt, K., Beer, A. E., and Grinnell, F. (1979). Disappearance of anionic sites from the surface of the rat endometrial epithelium at the time of blastocyst implantation. Biol Reprod 21, 691-707.

Hey, N. A., Graham, R. A., Seif, M. W., and Aplin, J. D. (1994). The polymorphic epithelial mucin MUC1 in human endometrium is regulated with maximal expression in the implantation phase. J Clin Endocrinol Metab 78, 337-42.

Hilkens, J., Vos, H. L., Wesseling, J., Boer, M., Storm, J., van der Valk, S., Calafat, J., and Patriarca, C. (1995). Is episialin/MUC1 involved in breast cancer progression? Cancer Lett 90, 27-33.

Hoffman, L. H., Olson, G. E., Carson, D. D., and Chilton, B. S. (1998). Progesterone and implanting blastocysts regulate Muc1 expression in rabbit uterine epithelium. Endocrinology 139, 266-71.

Huet-Hudson, Y. M., and Dey, S. K. (1990). Requirement for progesterone priming and its long-term effects on implantation in the mouse. Proc Soc Exp Biol Med 193, 259-63.

Humphrey, K. W. (1969). Induction of implantation of blastocysts transferred to ovariectomized mice. J Endocrinol 44, 299-305.

Jenkinson, E. J., and Searle, R. F. (1977). Cell surface changes on the mouse blastocyst at implantation. Exp Cell Res 106, 386-90.

Jones, C. J., Kimber, S. J., Illingworth, I., and Aplin, J. D. (1996). Decidual sialylation shows species-specific differences in the pregnant mouse and rat. J Reprod Fertil 106, 241-50.

Joziasse, D. H. (1992). Mammalian glycosyltransferases: genomic organization and protein structure. Glycobiology 2, 271-7.

Julian, J., Carson, D. D., and Glasser, S. R. (1992). Polarized rat uterine epithelium in vitro: responses to estrogen in defined medium. Endocrinology *130*, 68-78.

Kaneko, Y., Yamamoto, H., Colley, K. J., and Moskal, J. R. (1995). Expression of Gal beta 1,4GlcNAc alpha 2,6-sialyltransferase and alpha 2,6-linked sialoglycoconjugates in normal human and rat tissues. J Histochem Cytochem *43*, 945-54.

Kelm, S., Pelz, A., Schauer, R., Filbin, M. T., Tang, S., de Bellard, M. E., Schnaar, R. L., Mahoney, J. A., Hartnell, A., Bradfield, P., and et al. (1994). Sialoadhesin, myelin-associated glycoprotein and CD22 define a new family of sialic acid-dependent adhesion molecules of the immunoglobulin superfamily. Curr Biol *4*, 965-72.

Kemperman, H., Wijnands, Y., Wesseling, J., Niessen, C. M., Sonnenberg, A., and Roos, E. (1994). The mucin epiglycanin on TA3/Ha carcinoma cells prevents alpha 6 beta 4- mediated adhesion to laminin and kalinin and E-cadherin-mediated cell- cell interaction. J Cell Biol *127*, 2071-80.

Kimber, S. J. (1994). Carbohydrates and Implantation of the Mammalian Embryo. In Enocrinology of Embryo-Endometrium Interactions, S. R. Glasser, ed. (New York: Plenum Press), pp. 278-296.

Kimber, S. J., and Lindenberg, S. (1990). Hormonal control of a carbohydrate epitope involved in implantation in mice. J Reprod Fertil *89*, 13-21.

Kirk, A. T., and Murphy, C. R. (1990). Changes in intralysosomal environment in the uterine epithelium during early pregnancy in the rat. Acta Histochem *89*, 167-72.

Kitagawa, H., and Paulson, J. C. (1994). Differential expression of five sialyltransferase genes in human tissues. J Biol Chem *269*, 17872-8.

Knibbs, R. N., Goldstein, I. J., Ratcliffe, R. M., and Shibuya, N. (1991). Characterization of the carbohydrate binding specificity of the leukoagglutinating lectin from Maackia amurensis. Comparison with other sialic acid-specific lectins. J Biol Chem *266*, 83-8.

Kolinska, J., Baudysova, M., Zakostelecka, M., Kraml, J., and Kadlecova, L. (1990). Regulation of sialylation of intestinal brush-border enzymes and of sialyltransferase activity in organ cultures by dexamethasone. Biochem Int 22, 495-508.

Kolinska, J., Kraml, J., Zakostelecka, M., and Kadlecova, L. (1990). Actinomycin D suppresses hydrocortisone-induced changes in distribution and sialylation of brush-border enzymes in jejunal villi and crypts. Biochem Int 21, 581-91.

Kopitz, J., von Reitzenstein, C., Sinz, K., and Cantz, M. (1996). Selective ganglioside desialylation in the plasma membrane of human neuroblastoma cells. Glycobiology 6, 367-76.

Krehbiel, R. H. (1941). Anat Rec 81, 381-392.

Lammers, G., and Jamieson, J. C. (1989). Studies on the effect of lysosomotropic agents on the release of Gal beta 1-4GlcNAc alpha-2,6-sialytransferase from rat liver slices during the acute-phase response. Biochem J 261, 389-93.

Lasky, L. A. (1992). Selectins: interpreters of cell-specific carbohydrate information during inflammation. Science 258, 964-9.

Li, C., Tropak, M. B., Gerlai, R., Clapoff, S., Abramow-Newerly, W., Trapp, B., Peterson, A., and Roder, J. (1994). Myelination in the absence of myelin-associated glycoprotein. Nature 369, 747-50.

Ligtenberg, M. J., Buijs, F., Vos, H. L., and Hilkens, J. (1992). Suppression of cellular aggregation by high levels of episialin. Cancer Res 52, 2318-24.

Lindenberg, S., Kimber, S. J., and Kallin, E. (1990). Carbohydrate binding properties of mouse embryos. J Reprod Fertil 89, 431-9.

Lindenberg, S., Sundberg, K., Kimber, S. J., and Lundblad, A. (1988). The milk oligosaccharide, lacto-N-fucopentaose I, inhibits attachment of mouse blastocysts on endometrial monolayers. J Reprod Fertil 83, 149-58.

Ljungkvist, I. (1972). Attachment reaction of rat uterine luminal epithelium. IV. The cellular changes in the attachment reaction and its hormonal regulation. Fertil Steril *23*, 847-65.

Ma, J., and Colley, K. J. (1996). A disulfide-bonded dimer of the Golgi beta-galactoside alpha2,6- sialyltransferase is catalytically inactive yet still retains the ability to bind galactose. J Biol Chem *271*, 7758-66.

Ma, J., Qian, R., Rausa, F. M., 3rd, and Colley, K. J. (1997). Two naturally occurring alpha2,6-sialyltransferase forms with a single amino acid change in the catalytic domain differ in their catalytic activity and proteolytic processing. J Biol Chem *272*, 672-9.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory).

Marcus, G. J., and Shelesnyak, M. C. (1970). Steroids in nidation. In Advances in Steroid Biochemistry and Pharmacology, M. H. Briggs, ed. (New York: Academic Press), pp. 373-440.

Martin, C. R. (1985). The Female Reproductive System. In Endocrine Physiology (New York and Oxford: Oxford University Press), pp. 663-664.

Martin, L. (1984). On the source of uterine 'luminal fluid' proteins in the mouse. J Reprod Fertil *71*, 73-80.

Mattox, S., Walrath, K., Ceiler, D., Smith, D. F., and Cummings, R. D. (1992). A solid-phase assay for the activity of CMPNeuAc:Gal beta 1-4GlcNAc-R alpha-2,6-sialyltransferase. Anal Biochem *206*, 430-6.

McCormack, J. T., and Greenwald, G. S. (1974). Evidence for a preimplantation rise in oestradiol-17beta levels on day 4 of pregnancy in the mouse. J Reprod Fertil *41*, 297-301.

McCormack, J. T., and Greenwald, G. S. (1974). Progesterone and oestradiol-17beta concentrations in the peripheral plasma during pregnancy in the mouse. J Endocrinol *62*, 101-7.

Miller, R. L., Collawn, J. F., Jr., and Fish, W. W. (1982). Purification and macromolecular properties of a sialic acid-specific lectin from the slug Limax flavus. J Biol Chem *257*, 7574-80.

Milligan, S. R., and Martin, L. (1984). The resistance of the mouse uterine lumen to flushing and possible contamination of samples by plasma and interstitial fluid. J Reprod Fertil *71*, 81-7.

Morris, J. E., and Potter, S. W. (1984). A comparison of developmental changes in surface charge in mouse blastocysts and uterine epithelium using DEAE beads and dextran sulfate in vitro. Dev Biol *103*, 190-9.

Morris, J. E., Potter, S. W., and Gaza-Bulseco, G. (1988). Estradiol-stimulated turnover of heparan sulfate proteoglycan in mouse uterine epithelium. J Biol Chem *263*, 4712-8.

Negishi, H., Furuta, I., Ushigoe, K., Fujimoto, S., and Koide, S. S. (1998). In vitro production of progesterone and estradiol by rat granulosa cells regulated by cabergoline and prolactin. Endocr Res *24*, 195-203.

Nelson, J. D., Jato-Rodriguez, J. J., Labrie, F., and Mookerjea, S. (1977). Glycosyltransferase and UDP-galactose pyrophosphatase activities in the endometrium during oestrous cycle of the rat. J Endocrinol *73*, 53-8.

Nelson, J. D., Jato-Rodriguez, J. J., and Mookerjea, S. (1975). Effect of ovarian hormones on glycosyltransferase activities in the endometrium of ovariectomized rats. Arch Biochem Biophys *169*, 181-91.

Nicolson, G. L., Blaustein, J., and Etzler, M. E. (1974). Characterization of two plant lectins from Ricinus communis and their quantitative interaction with a murine lymphoma. Biochemistry *13*, 196-204.

Nilsson, B. O., and Hjerten, S. (1982). Electrophoretic quantification of the changes in the average net negative surface charge density of mouse blastocysts implanting in vivo and in vitro. Biol Reprod 27, 485-93.

O'Hanlon, T. P., and Lau, J. T. (1992). Analysis of kidney mRNAs expressed from the rat beta-galactoside alpha 2,6-sialyltransferase gene. Glycobiology 2, 257-66.

Owens, G. C., and Bunge, R. P. (1989). Evidence for an early role for myelin-associated glycoprotein in the process of myelination. Glia 2, 119-28.

Paria, B. C., Das, S. K., Andrews, G. K., and Dey, S. K. (1993). Expression of the epidermal growth factor receptor gene is regulated in mouse blastocysts during delayed implantation. Proc Natl Acad Sci U S A 90, 55-9.

Parr, M. B. (1983). Relationship of uterine closure to ovarian hormones and endocytosis in the rat. J Reprod Fertil 68, 185-8.

Parr, M. B., and Parr, E. L. (1977). Endocytosis in the uterine epithelium of the mouse. J Reprod Fertil 50, 151-3.

Parr, M. B., and Parr, E. L. (1989). The Implantation Reaction. In Biology of the Uterus, M. D. Wynn and W. P. Jollie, eds. (New York and London: Plenum Medical Book Company), pp. 233-277.

Parr, M. B., and Parr, E. L. (1974). Uterine luminal epithelium: protrusions mediate endocytosis, not apocrine secretion, in the rat. Biol Reprod 11, 220-33.

Paulson, J. C., Beranek, W. E., and Hill, R. L. (1977). Purification of a sialyltransferase from bovine colostrum by affinity chromatography on CDP-agarose. J Biol Chem 252, 2356-62.

Paulson, J. C., and Colley, K. J. (1989). Glycosyltransferases. Structure, localization, and control of cell type- specific glycosylation. J Biol Chem 264, 17615-8.

Peter, M. E., Dhein, J., Ehret, A., Hellbardt, S., Walczak, H., Moldenhauer, G., and Krammer, P. H. (1995). APO-1 (CD95)-dependent and -independent antigen receptor-induced apoptosis in human T and B cell lines. Int Immunol 7, 1873-7.

Pierce, M., Cummings, R. D., and Roth, S. (1980). The localization of galactosyltransferases in polyacrylamide gels by a coupled enzyme assay. Anal Biochem 102, 441-9.

Pimental, R. A., Julian, J., Gendler, S. J., and Carson, D. D. (1996). Synthesis and intracellular trafficking of Muc-1 and mucins by polarized mouse uterine epithelial cells. J Biol Chem 271, 28128-37.

Potter, S. W., Gaza, G., and Morris, J. E. (1996). Estradiol induces E-cadherin degradation in mouse uterine epithelium during the estrous cycle and early pregnancy. J Cell Physiol 169, 1-14.

Powell, L. D., Sgroi, D., Sjoberg, E. R., Stamenkovic, I., and Varki, A. (1993). Natural ligands of the B cell adhesion molecule CD22 beta carry N- linked oligosaccharides with alpha-2,6-linked sialic acids that are required for recognition. J Biol Chem 268, 7019-27.

Psychoyos, A. (1986). Uterine receptivity for nidation. Ann N Y Acad Sci 476, 36-42.

Raab, G., Kover, K., Paria, B. C., Dey, S. K., Ezzell, R. M., and Klagsbrun, M. (1996). Mouse preimplantation blastocysts adhere to cells expressing the transmembrane form of heparin-binding EGF-like growth factor. Development 122, 637-45.

Razi, N., and Varki, A. (1998). Masking and unmasking of the sialic acid-binding lectin activity of CD22 (Siglec-2) on B lymphocytes. Proc Natl Acad Sci U S A 95, 7469-74.

Rogers, G. N., and Paulson, J. C. (1983). Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. Virology 127, 361-73.

Sata, T., Roth, J., Zuber, C., Stamm, B., and Heitz, P. U. (1991). Expression of alpha 2,6-linked sialic acid residues in neoplastic but not in normal human colonic mucosa. A lectin-gold cytochemical study with Sambucus nigra and Maackia amurensis lectins. Am J Pathol *139*, 1435-48.

Schacher, H. (1994). Molecular cloning of glycosyltransferase genes. In Molecular Glycobiology, M. Fukuda and O. Hindsgaul, eds. (Oxford, New York and Tokyo: Oxford University Press), pp. 88-162.

Schachter, H. (1991). The 'yellow brick road' to branched complex N-glycans. Glycobiology *1*, 453-61.

Schauer, R. (1982). Chemistry, metabolism, and biological functions of sialic acids. Adv Carbohydr Chem Biochem *40*, 131-234.

Shibuya, N., Goldstein, I. J., Broekaert, W. F., Nsimba-Lubaki, M., Peeters, B., and Peumans, W. J. (1987). The elderberry (Sambucus nigra L.) bark lectin recognizes the Neu5Ac(alpha 2-6)Gal/GalNAc sequence. J Biol Chem *262*, 1596-601.

Shimamura, M., Shibuya, N., Ito, M., and Yamagata, T. (1994). Repulsive contribution of surface sialic acid residues to cell adhesion to substratum. Biochem Mol Biol Int *33*, 871-8.

Shur, B. D. (1991). Cell surface beta 1,4 galactosyltransferase: twenty years later. Glycobiology *1*, 563-75.

Smith, A. F., and Wilson, I. B. (1974). Cell interaction at the maternal-embryonic interface during implantation in the mouse. Cell Tissue Res *152*, 525-42.

Smith, D. M., and Biggers, J. D. (1968). The Oestrogen Requirement for Implantation and the Effect of its Dose on the Implantation Response in the Mouse. J Endocrinol *41*, 1-9.

Smith, S. E., French, M. M., Julian, J., Paria, B. C., Dey, S. K., and Carson, D. D. (1997). Expression of heparan sulfate proteoglycan (perlecan) in the mouse blastocyst is regulated during normal and delayed implantation. Dev Biol *184*, 38-47.

Strickland, S., and Richards, W. G. (1992). Invasion of the Trophoblasts. Cell *71*, 355-357.

Strous, G. J. (1986). Golgi and secreted galactosyltransferase. CRC Crit Rev Biochem *21*, 119-51.

Surveyor, G. A., Gendler, S. J., Pemberton, L., Das, S. K., Chakraborty, I., Julian, J., Pimental, R. A., Wegner, C. C., Dey, S. K., and Carson, D. D. (1995). Expression and steroid hormonal control of Muc-1 in the mouse uterus. Endocrinology *136*, 3639-47.

Taatjes, D. J., and Roth, J. (1988). Alteration in sialyltransferase and sialic acid expression accompanies cell differentiation in rat intestine. Eur J Cell Biol *46*, 289-98.

Tachi, S., Tachi, C., and Lindner, H. R. (1970). Ultrastructural features of blastocyst attachment and trophoblastic invasion in the rat. J Reprod Fertil *21*, 37-56.

Testa, J. E., and Quigley, J. P. (1990). The role of urokinase-type plasminogen activator in aggressive tumor cell behavior. Cancer Metastasis Rev *9*, 353-67.

Thie, M., Bochskanl, R., and Kirchner, C. (1986). Glycoproteins in rabbit uterus during implantation. Differential localization visualized using ^{3}H-N-acetyl-glucosamine labeling and FITC-conjugated lectins. Histochemistry *84*, 73-79.

Varki, A. (1994). Selectin ligands. Proc Natl Acad Sci U S A *91*, 7390-7.

Varki, A. (1997). Sialic acids as ligands in recognition phenomena. Faseb J *11*, 248-55.

Walmer, D. K., Wrona, M. A., Hughes, C. L., and Nelson, K. G. (1992). Lactoferrin expression in the mouse reproductive tract during the natural estrous cycle: correlation with circulating estradiol and progesterone. Endocrinology *131*, 1458-66.

Wang, X., Vertino, A., Eddy, R. L., Byers, M. G., Jani-Sait, S. N., Shows, T. B., and Lau, J. T. (1993). Chromosome mapping and organization of the human beta-galactoside alpha 2,6-sialyltransferase gene. Differential and cell-type specific usage of upstream exon sequences in B-lymphoblastoid cells. J Biol Chem *268*, 4355-61.

Wang, X. C., Smith, T. J., and Lau, J. T. (1990). Transcriptional regulation of the liver beta-galactoside alpha 2,6- sialyltransferase by glucocorticoids. J Biol Chem *265*, 17849-53.

Wasserman, D., and Wachbroit, R. (1992). The technology, law, and ethics of in vitro fertilization, gamete donation, and surrogate motherhood. Clin Lab Med *12*, 429-48.

Weinstein, J., de Souza-e-Silva, U., and Paulson, J. C. (1982). Purification of a Gal beta 1 to 4GlcNAc alpha 2 to 6 sialyltransferase and a Gal beta 1 to 3(4)GlcNAc alpha 2 to 3 sialyltransferase to homogeneity from rat liver. J Biol Chem *257*, 13835-44.

Weston, B. W., Nair, R. P., Larsen, R. D., and Lowe, J. B. (1992). Isolation of a novel human alpha (1,3)fucosyltransferase gene and molecular comparison to the human Lewis blood group alpha (1,3/1,4)fucosyltransferase gene. Syntenic, homologous, nonallelic genes encoding enzymes with distinct acceptor substrate specificities. J Biol Chem *267*, 4152-60.

White, S., and Kimber, S. J. (1994). Changes in alpha (1-2)-fucosyltransferase activity in the murine endometrial epithelium during the estrous cycle, early pregnancy, and after ovariectomy and hormone replacement. Biol Reprod *50*, 73-81.

Yamagata, T., and Yamazaki, K. (1991). Implanting mouse embryo stain with a LNF-I bearing fluorescent probe at their mural trophectodermal side. Biochem Biophys Res Commun *181*, 1004-9.

Yang, P., Major, D., and Rutishauser, U. (1994). Role of charge and hydration in effects of polysialic acid on molecular interactions on and between cell membranes. J Biol Chem *269*, 23039-44.

Yang, P., Yin, X., and Rutishauser, U. (1992). Intercellular space is affected by the polysialic acid content of NCAM. J Cell Biol *116*, 1487-96.

Yoshinaga, K., and Adams, C. E. (1966). Delayed implantation in the spayed, progesterone treated adult mouse. J Reprod Fertil *12*, 593-5.

Zhu, Z., Deng, H., Fenderson, B. A., Nudelman, E. D., and Tsui, Z. (1990). Glycosphingolipids of human myometrium and endometrium and their changes during the menstrual cycle, pregnancy and ageing. J Reprod Fertil *88*, 71-9.