Expression of L-selectin ligand MECA-79 as a predictive marker of human uterine receptivity

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Abstract

Problem Patients with repeated implantation failure (RIF) represent a subgroup of couples who suffer from unexplained infertility. Human blastocysts utilize L-selectin to initiate implantation by binding to endometrial ligands composed of oligosaccharide moieties on the surface glycoproteins. The absence of these ligands could lead to recurrent implantation failure (RIF) in some of these couples.

Methods Twenty fertile women and 20 patients with RIF were tested for the presence of the L-selectin ligands by immunohistochemistry. Endometrial biopsies were obtained on the sixth day post ovulation. After fixation, they were dated according to Noyes. Immunolocalization was performed using the MECA-79 antibody which is directed against ligands of L-selectin.

Results The fertile group all showed the presence of the L-selectin ligand. Of those with RIF, five were negative for the ligand and never, despite an average of five successive embryo transfers, became pregnant. Fifteen RIF

patients were positive for the L-selectin ligand, of whom ten subsequently conceived. As a screening test for RIF patients who lack the ligand, the predictive value was 100% with a sensitivity of 50% and specificity of 100%. The positive predictive value was 100% and negative predictive value is 87%.

Conclusions L-selectin and its ligands play a vital role for early human implantation. Screening for the absence of the ligand may help many patients with RIF to avoid undergoing repeated failed treatment cycles.

Keywords Human implantation · L-selectin ligand · MECA-79 · Blastocyst

Introduction

During implantation and placentation, a human embryo must attach itself to the uterus under conditions of shear stress. After passing down the fallopian tube, the embryo is moved within the uterine lumen by rhythmic myometrial contractions until it can physically attach itself to the endometrial epithelium. This process is a complex series of events that can be divided into three distinct steps: apposition, attachment, and invasion [1]. Once the human blastocyst hatches from the zona pellucida, the free-floating sphere of cells must orient itself as it approaches the endometrial surface and form an initial adhesion (apposition) before it can firmly attach and begin the process of invasion. The apposition step is a transient and dynamic process. Uterine contractions and mucin secretion within the uterine cavity propel the blastocyst around the cavity. Despite these fluid dynamics which creates shear stress, the embryo is able to approach the wall, roll around to right itself so that the trophoectoderm overlying the inner cell

Absence of the L-selectin ligand on the endometrium acts as a predictive marker for failed implantation.

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mass apposes to the endometrial surface. Shortly after the apposition step, an integrin-dependent adhesion occurs. This allows the blastocyst to attach firmly to the uterine wall and trophoblasts transmigrate across the luminal epithelium, burying the embryo beneath the uterine wall.

The implantation process bears some similarity to leukocyte transmigration across the blood vessel wall. In the vasculature, under shear flow, leukocytes must use specialized mechanisms that enable them to first adhere and subsequently transmigrate across endothelial layer of the blood vessel wall. Numerous studies [2–4] have shown that the initial interactions between leukocytes and vascular endothelial cell surface are mediated by selectin adhesion systems. Selectins are lectin-like proteins that include E-, L- and P-selectins, all of which were originally thought to be expressed exclusively by hemangioblast descendants. E-selectin is expressed on activated endothelial cells, P-selectin is expressed on the surfaces of activated platelets and endothelial cells and L-selectin is expressed on lymphocytes. These lectin-like molecules recognize specific oligosaccharide structures carried on some glycoproteins including PSGL-1, CD34, GlyCAM-1, MAdCAM-1, podocalyxin and endoglycan. Recently, it has also been shown that salivary mucins bear specific carbohydrate motifs that can serve as ligands for L-selectins [5, 6]. The selectin adhesion systems allow leukocytes to tether and roll on the endothelial surface [2]. These types of interactions have rapid and reversible properties. Traveling leukocytes slow down, tether and roll on the epithelial cell surface. Once arrested, integrin activation triggers stable adhesion (shear-resistant) and allow subsequent transmigration of leukocytes through the vascular endothelium [2, 3].

At the morphological level, there are parallels between leukocyte extravasation from the vasculature and attachment of embryo to the uterine wall since both types of adhesion occur under shear flow and are followed by integrin activation. Genbacev et al. [7] have recently shown that the L-selectin adhesion system plays a crucial role during an initial step of blastocyst implantation. Hatched blastocyst expresses L-selectin and uses this molecule to mediate its attachment to the luminal epithelial surface via its carbohydrate ligands, MECA-79 and related epitopes. Two different *in vitro* models illustrating L-selectin mediating adhesion were used to support the proposal. In the first model, beads coated with synthetic L-selectin carbohydrate ligands were overlaid on placental chorionic villous explants under shear stress. It was shown the beads bound to cell column cytotrophoblasts (CTBs) on the explants and the binding was blocked by antibody to L-selectin. Additional immunolocalization experiment confirmed that the CTBs did express L-selectin. In the second model, isolated CTBs were overlaid on endometrial tissue sections under shear stress. It was shown that CTBs bound to

epithelial surface of the endometrium tissues that were obtained during the luteal phase and did not bind to those that were obtained during the follicular phase. Again, the binding was blocked by anti-L-selectin antibody. Concurring with these observations, immunolocalization study of endometrial tissue demonstrated that the L-selectin carbohydrate ligand MECA-79 was unregulated from the day of ovulation to 6 days post ovulation, and was negative throughout the follicular phase and remained negative if ovulation did not occur. More recently, Carson et al. [8] have shown that MUC-1, a transmembrane mucin glycoprotein expressed at the apical surface of the uterine epithelia, likely serves as a scaffold for the L-selectin carbohydrate ligands. Together, these studies suggests that the human embryo uses a mechanism well studied in leukocytes to mediate rolling and tethering onto the endometrial wall prior to firm adhesion when integrins begin their crucial role.

Given the many and varied causes of infertility and early pregnancy loss, we hypothesized that defects in the selectin adhesion system could account for a portion of unexplained reproductive failures. In practice, there are subgroups of women undergoing *in vitro* fertilization who repeatedly fail to implant despite the transfer of top quality embryos. Our goal was to assess whether the absence of the L-selectin ligand MECA-79 on the endometrium occurs more frequently in patients with repeated implantation failure (RIF), and if the lack of the endometrial L-selectin ligand correlates with unsuccessful implantation.

Materials and methods

Study design

We compared the presence of endometrial L-selectin ligand in a fertile population to a subgroup of patients with a history of repeated implantation failures. The subjects underwent endometrial biopsies during the mid-luteal phase (e.g. implantation window) of an ovulation induction cycle. Those patients with implantation failure continued their pursuits of pregnancy through further treatments. The outcome of each patient was followed and tabulated according to whether they did or did not have the L-selectin ligand recognized by immunostaining with the antibody MECA-79.

Patient selection

Control subjects were healthy young women with proven fertility who were serving as anonymous ovum donors. Twenty RIF patients were recruited at the Nevada Center for Reproductive Medicine from April 2003 to October

Fig. 1 Presence of L-selectin ligand MECA-79 immunostaining among patients

2006. Approval for this research was obtained from the Washoe Medical Center Institutional Review Board of the Committee for Human Rights in Research. Patients were recruited if they had at least two unsuccessful in-vitro fertilization cycles due to implantation failure. The diagnosis of implantation failure was made if no implantation occurred despite the transfer of top-quality embryos into a normal appearing endometrial cavity. The embryo morphology was scored according to criteria by Veeck [9]. Only patients who had 6–8 cell stage embryos, grade 1 and 2 transferred were included. The uterine cavity was assessed pre-cycle by sonohysterography and in cycle by transvaginal sonographic evaluation of the endometrial thickness and appearance. Only patients with an endometrial thickness greater than 7 mm and a trilaminar appearance were included.

Endometrial biopsy

Informed consent was obtained from all patients according to the IRB protocol approved by the Washoe Medical

Center Committee on Human Research. The phase of menstrual cycle was confirmed by either a controlled stimulation cycle or a natural cycle monitoring. The stimulation comprised 14 days of estradiol (estrace 2 mg orally bid), followed by micronized progesterone (prometrium 200 mg orally bid) plus estradiol (estrace 2 mg orally qd). In the natural cycles ovulation was confirmed by both a late follicular ultrasound and LH monitoring. An endometrial biopsy was obtained on day 6 of progesterone or the seventh day post LH surge by advancing a Unimar pipelle to the fundus, creating negative pressure and pulling the catheter out in a spiral fashion. The tissue was immediately fixed in 3% paraformaldehyde for 24 h. It was rinsed three times in PBS, infiltrated with 5 to 15% sucrose followed by OCT compound and frozen in liquid nitrogen. The tissue was sectioned (5 μ m) using a cryomicrotome (Leica Microsystems, Bannockburn, IL) for immunolocalization.

Endometrial dating

The morphology of the endometrial biopsies was scored according the dating criteria of Noyes et al. [10] by an experienced histologist. The histologist was blinded to the patient history, including the cycle day of endometrial biopsy.

Immunohistochemistry

Rat monoclonal antibodies that recognize L-selectin ligand, MECA-79, were from BD Biosciences, San Jose, CA. The MECA-79 antibody recognizes a high-affinity L-selectin ligand carbohydrate epitope containing SO₃→6GlcNAc [11]. The primary antibody was added at a concentration of 5 μ g/ μ l. After the specimens were incubated at 4°C overnight, they were washed three times in PBS and incubated with goat FITC-conjugated anti-rat IgM (Jackson ImmunoResearch Laboratories). As controls, an irrelevant

Table 1 Summary of patients with repeated implantation failure

L-selectin ligand (N)	Age (SD)	Uterine history	IVF history (no. of cycles)	Implantation rate (pregnancies/no. embryos)
Absent (5)	39.0 (1.67)	Unicornuate (2) Asherman's syndrome Adenomyosis Many myomectomies	25/5 (5)	0/72
Patchy (4)	34.7 (4.43)	Normal (1) Hydrosalpingectomy (2) Postpartum curettage	17/4 (4.25)	3/47 (15.7%)
Normal (11)	40.1 (6.73)	Normal (6) Myomectomy (2) Mural myomas (2) Uterine septoplasty	40/11 (3.6)	11/76 (14.5%)

Table 2 L-selectin ligand and outcome in RIF patients

Patients	L-selectin ligand	Implantation	Normal uterus	Implantation rate (%)	Mean number of cycles
5	Absent	None	0/5	0/72 embryos	5
10	Present	10	7/10	14/116 (12.1%); after endometrial biopsy, 14/57 (25%)	4.3
5	Present	None	1/5	0/37	2.8

rat IgM antibody (anti-KLH, eBioscience) or PBS was substituted for primary antibody. Staining was evaluated by using a Zeiss Axiophot fluorescence microscope.

Results

First we tested the expression of MECA-79 in the endometrial biopsies from control group that consisted of 20 healthy women who were proven to be fertile. All biopsy specimens had morphological characteristics of mid-luteal phase endometrium. The immunostaining with MECA-79 antibody revealed that this L-selectin ligand was present in all samples from the control group and that positive immunostaining was associated with the surface of the luminal and glandular epithelium. Interestingly, one control patient who initially stained negative for MECA-79 was found to be out of phase during a natural cycle. Later we discovered she had not ovulated. She was placed on a controlled stimulation cycle and subsequently stained positive at the mid-luteal phase.

The group of RIF patients was very heterogeneous. The immunostaining with MECA-79 was positive in 15 out of 20 specimens (i.e., in 75% of examined biopsies—see Fig. 1). Of these 15 positive specimens, 4 exhibited weak or patchy staining and 11 were normal. All five MECA-79 negative samples were associated with severe uterine anomalies, including congenital anomalies (i.e. unicornuate uterus), Asherman's syndrome, adenomyosis and multiple myomectomies. None of these patients became pregnant after an average of five separate attempts of embryo transfer (see Table 1). Out of 15 patients with MECA-79 positive biopsies, 10 became pregnant and 5 quit treatments after an average of one more transfer following the biopsy. Of

those that continued, the majority became pregnant over an average of two more transfers (see Table 2). Those patients that were positive for the ligand, but did not conceive stopped their treatments an average of 1.5 cycles sooner than those that did get pregnant (2.8 vs 4.3 cycles respectively). There were no complications with the pregnancies to date.

Discussion

The results of this pilot study have some important implications. First, the lack of expression of L-selectin ligand MECA-79 in the mid-luteal endometrial biopsy specimen in this group of patients was indicative of a very low or no chance of pregnancy. The predictive value was 100% with a sensitivity of 50% and specificity of 100% (see Table 3). Undoubtedly, the sensitivity would improve if the patients that quit treatments had persisted and became pregnant. Second, patients with a positive MECA-79, that had failed two good prognosis cycles, had about 32% chance per cycle to achieve pregnancy subsequently. Third, in eight patients with normal uterus and positive immunostaining with MECA-79 the probability of pregnancy was higher than 85% cumulatively, or 53.8% (7:13) per cycle. The implantation rate of those who became pregnant, however, was only 12% overall which is less than one third the rate seen for all patients at the treatment center. Regardless of the presence of the L-selectin ligand, these patients clearly represent a sub-fertile group.

The apical surface of the endometrium contains key elements for the initiation of molecular interactions to capture the human blastocyst. The endometrium becomes hormonally primed through the menstrual cycle to create a

Table 3 Predictive value of L-selectin ligand absence to the lack of a subsequent pregnancy (screening test performed after at least two failed cycles)

L-selectin ligand	Not pregnant	Pregnant	Positive predictive value	Negative predictive value	RIF pts, subsequent cycles	Pregnancy per cycle (%)
Absent	5	0	100%		15	0/15
Present	5	30		86%	31	10/31 (32.2)
Sensitivity of test	50%					
Specificity of test		100%				

period of optimal receptivity to successful embryonic implantation. This “window of implantation” occurs between days 20 to 26. Outside this window, the endometrium is resistant to embryo attachment [12]. Lai et al. [13, 14] described the expression of L-selectin ligand throughout the natural menstrual cycle and controlled ovarian stimulation cycle. Its expression increased from the periovulatory interval to the mid-secretory phase. Peak immunostaining for L-selectin ligand was seen at the early to midsecretory interval on the luminal surface which coincides with the window of implantation. Interestingly, a reduction in expression was seen in subjects who received ovulation induction medication. Vlahos et al. [15] found that progesterone supplementation enhances L-selectin ligand expression in the luteal phase following controlled ovarian stimulation. Preliminary work by Khan et al. [16] found that N-acetylglucosamine-6-O-sulfotransferase (GlcNAc-6-OST), the gene responsible for high affinity L-selectin ligand epitope production, is regulated by estrogen and progesterone. Estrogen up regulates the gene, while progesterone amplifies this action. Progesterone alone however will suppress GlcNAc -6-OST expression, presumably rendering the endometrium non-receptive.

In our study group, those patients who lacked the ligand all had high risk histories for uterine defects. Iatrogenic causes include curettage and myomectomy while natural states like a congenital anomaly can also give rise to endometrial defects. Clearly, not all patients with such histories fail to achieve pregnancy, but perhaps the L-selectin ligand MECA-79 may act as a marker for the extent of injury or anomaly prior to attempts at pregnancy in high-risk groups.

There could be other disease states that affect the expression of L-selectin ligand. Lessey [17] described aberrant integrin expression in the endometrium of women with endometriosis. Similarly, Kao et al. [18] found the gene, GlcNAc-6-OST, is down-regulated in patients with endometriosis. Mak et al. [19] found androgens suppress the gene expression which may play a role in poorer reproductive outcomes among patients with polycystic ovarian syndrome.

Recently, Shamonki et al. [20] correlated L-selectin ligand expression with the pregnancy rate in subsequent donor egg cycles. They demonstrated significantly higher immunohistochemical reactivity for the L-selectin ligand at the apex of endometrial surface epithelium obtained during mock cycle from donor egg recipients who subsequently conceived compared to those who did not. They scored the intensity of staining and correlated it to pregnancy rate, rather than the presence or absence as we have done. The study further supports our finding that L-selectin plays a role in implantation not only by its presence but also by its degree.

We demonstrate that the absence of L-selectin ligand in patients with multiple failed implantation will continue to

fail further attempts at implantation. Those who have failed but test positive for the L-selectin ligand, have a very good prognosis on subsequent trials of implantation despite having other unknown contributors to their subfertility.

Conclusions

The L-selectin ligand adhesion system is becoming more convincingly believed to play a major role in mediating initial embryonic apposition. By loose tethering, the blastocyst is able to attach despite the shear forces within the uterine lumen and orient itself for stable attachment. Once anchored, a cascade of events unfolds allowing the embryo to burrow into the endometrial wall and establish a hemochorial placenta. Clinically, we may use L-selectin ligand as a marker for implantation efficiency. Clearly, in high risk groups, one could biopsy a patient and potentially prevent many futile attempts of costly treatments and provide the patient with important information as to why she is unable to become pregnant. Further studies must be done to establish criteria sets for testing appropriate high-risk groups and to develop a treatment design.

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