

Assessment of Leukemia Inhibitory Factor and Glycoprotein 130 Expression in Endometrium and Uterine Flushing: A Possible Diagnostic Tool for Impaired Fertility

Manal Mostafa, Manal Eid*, Hesham El-Sorogi*, Manal Abdallah*, Azza Hasan**

*Department of Gynecology and Obstetrics, Tanta University, *Department of Clinical Pathology, Tanta University, **Department of Microbiology, Tanta University*

ABSTRACT:

Background/Aim: uterine receptivity and implantation are complex processes requiring coordinated expression of molecules by the embryo and uterus during implantation. This article focuses on the endometrial expression of LIF and its gp130 receptor molecules and their secretion in uterine flushing during the window of implantation in cases of primary unexplained infertility.

Patients & Methods: The study was conducted on 25 infertile women with unexplained infertility for at least 2 years and 10 normal fertile women as a control group. Endometrial tissue and uterine flushing were obtained, each tissue specimen was divided into two pieces; one piece was used for histological dating of the endometrium and used also for immunostaining of progesterone receptor, and the second piece was used for RNA extraction and PCR assay of LIF and gp130 mRNA expression. Venous blood samples were collected from all women participating in the study for measurement of serum estrogen and progesterone.

Results: LIF mRNA was expressed in the endometrium of all normal fertile women but significantly decreased in infertile women. LIF was not detectable in 88% of infertile women and fairly detectable in 12% of them. gp130 mRNA was hardly detectable in both fertile and infertile women with no difference between the two groups. Infertile women secreted significantly less LIF and gp130 molecules in the uterine flushing compared to normal fertile women.

Conclusion: Expression of LIF mRNA in endometrium could be used as a molecular marker of unexplained infertility. Assessment of secreted LIF and gp130 molecules in uterine flushing could be another useful and safe method for predicting successful implantation as well as for diagnosing and eventually treating women with impaired fertility using recombinant human LIF.

Key words: Leukemia Inhibitory Factor, Glycoprotein 130 Expression, Endometrium, Uterine Flushing, Impaired Fertility.

INTRODUCTION

Of the 1/10 couples having problems conceiving 25% have unexplained infertility⁽¹⁾. Embryo implantation is a critical step in establishment of pregnancy. Implantation is the

process by which the blastocyst becomes intimately connected with the maternal endometrium (decidua). In humans, the process of implantation can be divided into three

phases: apposition, adhesion, and invasion. The apposition comprises the blastocyst orientation in the uterine cavity towards the endometrium. During the adhesive and invasive phases, the blastocyst approaches the epithelium, attaches itself to it and the embryo trophoblast invades the deciduas. All these steps are controlled by a variety of interacting molecules of both maternal and embryonic origin⁽²⁾. Many cytokines, growth factors and adhesive molecules are known to trigger the initial process of implantation during the adhesive phase, and accomplish the embryo-maternal contacts during the invasive phase.

It is well known that the endometrium becomes receptive for a limited period of time after exposure to 17- β -estradiol (E) followed by progesterone. Embryo transfer studies have identified a phase of uterine receptivity, 'the window of implantation', between days 5 and 10 following the luteinizing hormone (LH) surge⁽³⁾. Many factors produced by the endometrium during the 'the window of implantation' have been proposed to be molecular markers of a receptive endometrium. While progesterone receptor is the only factor as yet identified to be absolutely required for successful implantation, leukemia inhibitory factor (LIF) and mucin1 are regarded as two of the most important signaling vectors⁽⁴⁾.

Leukemia inhibitory factor is a pleiotropic cytokine of the IL-6 family, meaning that it has effects on many different cell types and that its activities are not restricted to one lineage⁽⁵⁾. It is a polyfunctional highly glycosylated protein (Mw 38-67 KDa) that mediates a wide range of effects in different ways, from promotion of cell proliferation and lifespan, to the control of their differentiation in regard to the tissue environment in which the target cells perform their functions. LIF mature protein of maternal origin and its encoding mRNA have been identified in endometrium, fallopian tubes and cervical mucus. In the endometrium, LIF expression level remains low during the proliferative phase, rises after ovulation and remains high until the end of each menstrual cycle with its maximal expression during the mid-late secretory phase. Its high endometrial production in the mid and late phase of

menstrual cycle stresses its important role in implantation. Simultaneously, the blastocyst expresses LIF receptor (LIF-R); this documents the significance of LIF in the embryo-maternal crosstalk. LIF-R is also expressed on the endometrium and oocyst⁽⁶⁾. Other cells expressing LIF receptors include neurons, megakaryocytes, macrophages, adipocytes, hepatocytes, osteoblasts, myeloblasts, and kidney and breast epithelial cells. LIF receptor complex includes two subunits, the LIF-specific subunit LIFR- β and the gp130 subunit (also used by IL-6 and IL-11). Glycoprotein 130 subunit is a transmembrane protein. Soluble forms of gp130 are generated by proteolytic cleavage or by alternative splicing. Binding of LIF to its receptor promotes formation of a receptor complex with gp130. Signal transduction involves activation of members of the Janus family and phosphorylation of members of the STAT family of transcription factors. The LIFR- β /gp130 heterodimer can bind and signal in response to oncostatin M, ciliary neurotrophic factor, and cardiotrophin, in addition to LIF. Binding of LIF to LIF receptor and gp130 activates signal transduction pathways^(5,7).

In human, LIF can be detected in endometrial biopsy as well as in uterine flushing. The majority of women with unexplained infertility had dysregulated LIF production. Likewise, endometrial explants derived from infertile women showed reduced levels of LIF secretion⁽⁸⁾. Based on these findings the study was designed to investigate the expression of leukemia inhibitory factor and its receptor subunit gp130 in endometrium of infertile women.

SUBJECTS AND METHODS

Patient selection

The study was conducted on 25 infertile women with unexplained infertility for at least 2 years and 10 normal fertile women as a control group. The inclusion criteria for the patients was 2 years of primary unexplained infertility which was diagnosed by the following presence of regular menstrual cycle between 21 and 35 days,

absence of tubal or ovarian pathology as proved by laparoscopy and hysterosalpingography, absence of endometriosis proved by laparoscopy, absence of ovulatory disorders proved by folliculometry and normal serum progesterone level, normal semen analysis according to WHO criteria (9), normal thyroid function and normal plasma prolactin concentration. The mean age of the infertile women was 31.1 ± 4.2 years (range 24-39 years). The mean age of the fertile women was 30.7 ± 6.5 years (range; 20-38 years). Each of the fertile women had at least one live birth, without history of infertility or miscarriage. None of the participants had steroid or other medications for at least 2 months prior to collection of samples.

Materials

Endometrial tissue and uterine flushing were obtained from women who gave informed consent. Endometrial tissue was (obtained by) collected by obtaining small strips of endometrium using Novak's curette. Uterine flushing was performed as an outpatient procedure as described previously (9). A bivalve speculum was inserted, through which a size 8 Foley catheter was introduced into the uterine cavity and the balloon of the catheter was inflated with 1 ml normal saline. 2 ml of saline were gradually flushed into the uterine cavity. Afterwards, gentle suction was applied to recover the fluid. The tissue and flushing samples were stored at -20°C for LIF and gp130 assays.

All specimens were collected during the implantation window. (LH surge LH+6 to LH+11)⁽⁷⁾. Each tissue specimen was divided into two pieces; one piece was used for obtaining tissue sections for histological dating of the menstrual cycle by a pathologist according to the method of Noyes et al. (10) and used also for immunostaining of progesterone receptor, and the second piece was used for RNA extraction and PCR assay of LIF and gp130 mRNA expression. Uterine flushing was used for estimation of LIF and gp130 secretion by ELISA assay. Venous blood samples were collected from all women participating in the study for measurement of serum estrogen and progesterone.

Assessment of mRNA expression of leukemia inhibitor factor and gp130 in endometrium by PCR

RNA extraction and cDNA synthesis

Total cellular RNA was extracted from endometrial tissue using Qiagen RNeasy mini spin column (RNeasy Mini Kit, Qiagen, USA) according to the manufacturer protocol. Complementary DNA was prepared from the RNA as follows: 2 μg of total RNA was reverse-transcribed with random hexamers by use of a commercial kit (High-Capacity cDNA kit, Applied Biosystems, USA) under the following conditions: hexanucleotides annealing for 10 min at 25°C , cDNA synthesis for 30 min at 48°C , followed by enzyme inactivation for 5 min at 95°C .

cDNA amplification

cDNA was used as a template to amplify LIF and the two splice variants of LIF receptor subunit gp130. The amplification mixture was performed in a final volume of 50 μl containing 5 μl cDNA, 25 μl Taq PCR master mix (2.5 units Taq DNA polymerase, 1x PCR buffer, and 200 μM of each dNTP) (Taq PCR master kit, Applied Biosystem, USA), and 200 μM of each primer. PCR conditions for used an initial denaturation at 95°C for 5 minutes followed by 35 cycles of: 1 minute denaturation at 94°C , 1 minute annealing at 63°C , and 1 minute extension at 72°C , followed by a final extension at 72°C for 5 minutes (for LIF), 30 minute denaturation at 94°C , 30 minute annealing at 57°C , and 1 minute extension at 72°C , followed by a final extension at 72°C for 5 minutes (for gp130 A and B). Amplification of gp130 using primers C and D was performed in the same way except that the primer annealing temperature was 50°C (7,11). As an internal control, GAPDH was amplified to justify for difference in RNA input and reverse transcription efficiency. Amplification of GAPDH was performed for each sample in a separate tube using the following primers and probe forward primer: 5'-GAAGGTGAAGGTCCGAGTC3', reverse primer: 5'-GAAGATGGTGATGGGATTC3'. PCR conditions for GAPDH were an initial denaturation at 95°C for 5 minutes followed by

35 cycles of 45 seconds denaturation at 94°C, 45 seconds annealing at 62°C, and 1 minute extension at 72°C, followed by a final extension at 72°C for 5 minutes. The amplified DNA was fractionated on 1% agarose gel and photographed. The density of the DNA bands was measured. The density of LIF and gp130 bands was divided by the density of GAPDH of the same sample to get the normalized value of their expression.

Measurement of LIF and glycoprotein 130 in uterine flushing

Levels of LIF and gp130 were measured in uterine flushing using enzyme-linked immunosorbent assays (ELISA). LIF was measured using LIF ELISA kit, Bender Medsystems, USA and gp130 was estimated using Quantikine ELISA kit, R&D Systems Inc. The sensitivity of the LIF assay ranges from 0.45-500 pg/ml and that for gp130 ranges from 40-6000 pg/ml. USA. The procedures were performed according to the manufacturer instructions and fresh saline was used as zero standards in both estimates.

RESULTS

Expression of LIF and gp130 mRNA in endometrium

Leukemia inhibitory factor and the 2 gp130 splice variants mRNA was measured in endometrial tissue taken from fertile and infertile women during the implantation window (LH surge LH+6 to LH+13) using RT-PCR. The fertile and infertile women enrolled in the study were matched as regards their age, hormonal profile in plasma (FSH, LH, estrogen, progesterone and prolactin), and endometrial expression of progesterone receptor (table 2, figure1).

LIF expression in endometrium showed a significant difference between fertile and infertile women ($P < 0.001$). LIF expression was

significantly low in infertile compared to fertile women. All fertile women showed marked LIF mRNA expression in endometrium. Three (12%) only of the 25 infertile women enrolled in the study showed detectable endometrial LIF expression and twenty two (88%) patients did not show any LIF mRNA in their endometrial samples.

RT-PCR analysis of the two gp130 splice variants showed very faint expression in both fertile and infertile women with no difference between the two groups ($P > 0.05$). In fertile group, 3 (30%) women did not show any gp130 splice variant expression and 7 (70%) women showed very low expression of splice variant. Similarly, 6 (24%) of the 25 infertile women showed complete absence of gp130 mRNA expression and 19 (76%) showed very faint gp130 variant1 (figure1).

Secretion of LIF and gp130 in uterine flushing

Uterine flushing was performed using 2 ml saline. The procedure was done very gently to avoid pain. Absence of pain is an essential feature of this exploration since pain may enhance cytokine production and thereby produce inconsistent results. Estimation of LIF and gp130 concentration in flushing was performed using the sensitive ELISA assay (table2).

Secretion of LIF in uterine flushing was significantly lower in infertile women compared to fertile women ($P < 0.001$). In fertile women, uterine flushing LIF level ranged from 18-120 pg/ml (mean: 48.8 ± 28.9). In infertile women, uterine flushing LIF level varied between 0.5 and 35 pg/ml (mean 3.9 ± 7.5). Similarly, secretion of gp130 in uterine flushing was significantly lower in infertile women group (mean 51.5 ± 27.5 , range; 25-140 pg/ml) compared to fertile group (mean: 182 ± 77 , range; 95-370 pg/ml) ($P < 0.001$) (table2).

Table 1: primer sequences used in the study (7,11)

Primer	Location	Sequence(5' to3')
LIF sense	3174-3195	CAGCATCACTGAATCA CAGAGC
LIF antisense	3712-3733	CCCTGTGGGATGTTT CATACT
Splice variant 1		
gp130 A	927-946	ATACTGGAGTGACTGGAGTG
gp130 B	1099-1118	CATCITGTGAGAGT CACITC
Splice variant 2		
gp130 C	1767-1790	GGTACGAA TGGCAGCATA CA
gp130 D	2480-2461	CTGGACTGGATT CATGCTGA

Table 2: comparison of plasma hormones, endometrial progesterone receptor and LIF and gp130 expression in endometrium and uterine flushing in fertile and infertile women

	Fertile	Infertile	Significance, P
Age (mean±SD)	30.7±6.5	31.1±4.2	>0.05
Prolactin (mean±SD)	13.1±5.6	11.7±4.4	>0.05
FSH in Serum	5.6±1.8	6.9±1.4	>0.05
LH in serum	9.3±4.0	8.1±5.1	>0.05
Estradiol in serum	9.1±3.3	10.5±4.0	>0.05
Progesterone in serum	7.1±5.1	6.0±4.8	>0.05
Progesterone receptor in endometrium	Expressed in 100%	Expressed in 100%	>0.05
LIF			
- In uterine flushing	48.8±28.9	3.9±7.5	<0.001*
- In endometrium	Expressed in 100%	Expressed in 12%	<0.001*
gp130			
- In uterine flushing	182±77	51.5±27.5	<0.001*
- In endometrium	Expressed in 70%	Expressed in 76%	>0.05

* denotes statistical significance.

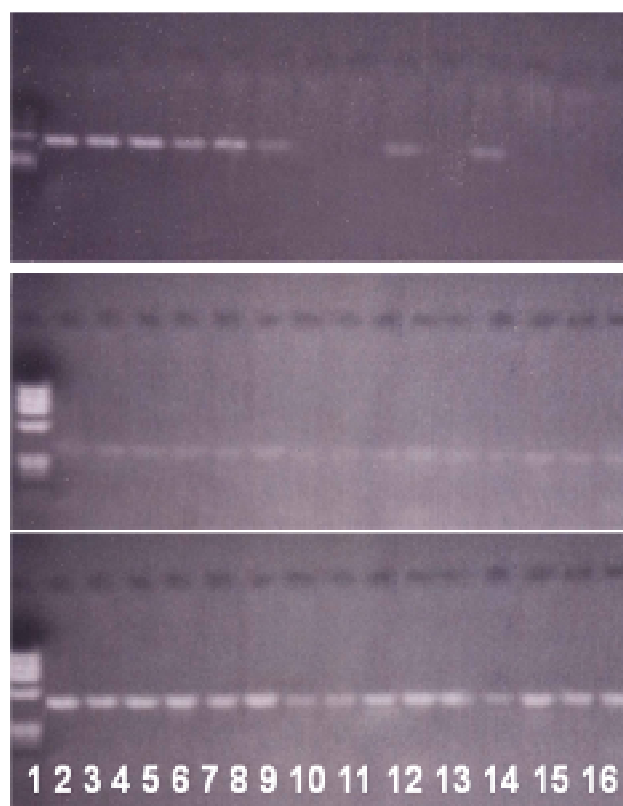


Figure 1: Expression of LIF and gp130 splice variant 2 in endometrium of fertile and infertile women.

The upper figure represents LIF expression, middle figure represents expression of gp130 and the lower one is the GAPDH expression. Lane 1 is the DNA ladder (100 bp), lanes from 2-5 are fertile women and lanes 6-16 are infertile women.

DISCUSSION

The implantation process is currently considered as the most relevant limiting factor for successful pregnancy. Molecules that affect uterine receptivity for subsequent implantation of the blastocyst include integrins, colony stimulating factor-1 (CSF-1) and leukemia inhibitory factor (LIF). The fact that LIF is the key molecule in the implantation process in monkey and mice inspired similar research in humans. Human studies demonstrated that LIF mRNA is present in endometrium and that the rise in expression of LIF coincides with the implantation window. LIF belongs to the IL-6 like family of cytokines that signal through specific receptors on the cell surface that all

share gp130 subunit as a common accessory signal transduction molecule (12).

The role of endometrial LIF in implantation has been documented in animals and human. LIF has been also found in the fallopian tube during preimplantation period indicating a role for LIF in communication between the embryo and the tube. This observation suggests that absence of LIF in the mother, not in the embryo, is responsible for failure of implantation (13,14). We started our study by confirming the expression of LIF mRNA in the endometrium of both fertile and infertile women during the implantation window. LIF mRNA expression in the endometrium was significantly reduced in

infertile than fertile women. Dimitriadis et al.,⁽¹⁵⁾ reported that immunostaining of endometrial tissue from fertile and infertile women for the molecules affecting implantation, IL-11, IL-11R α , and LIF, demonstrated equal staining for IL-11 and its receptor, but marked reduction of LIF staining in infertile women than fertile group. LIF gene mutations have been described in nulligravida infertile women, and have been hypothesized that they might lead to transcription abnormalities and decreased LIF expression^(12,16).

The validity of measuring LIF secretion in the fluid of uterine flushing as a non invasive technique enabling determining that a low LIF concentration in such fluid during late luteal phase could be predictive of implantation failure was documented⁽¹⁵⁾. The present study showed that secretion of LIF in uterine flushing of infertile women during window of implantation was markedly low. Several previous studies reported lower LIF in uterine flushing in women with primary unexplained infertility compared to fertile women, and lower LIF secretion from endometrial explants of infertile women than fertile ones especially during the implantation window^(8, 15,17). These results are not conflicting with the observation by Ledee-Bataille et al.,⁽¹⁸⁾ who showed an inverse correlation between uterine flushing LIF concentration and the likelihood of successful implantation. He might use different ELISA kit. It has been reported before that commercially available ELISA kits are far from equivalent. The kits mainly differ in their ability to detect glycosylated protein. Human LIF is known to be highly glycosylated and sugar moiety represents as much as 50% of the total weight of the naturally produced protein. Several peptide epitopes may therefore be masked and the protein may not be detectable by the antibodies in certain kits⁽¹²⁾.

In fact, presence of sufficient amount of LIF protein seems to be an essential condition for implantation, but a variety of other regulatory mechanisms, such as malfunction of LIF receptor, IL-6 signal transducer (gp130), which is an affinity modulator for the LIF protein receptor complex⁽¹²⁾. In a previous study,

soluble gp130 was the most abundant member of the IL-6 cytokine-receptor family secreted by the endometrium at the time of implantation⁽⁷⁾. Therefore, the present study focused on exploration of gp130 status in both endometrium and uterine flushing. The study showed minimal expression of gp130 splice variant1 in the endometrium of both fertile and infertile women equally. However, infertile women secreted significantly reduced levels of soluble gp130 in uterine flushing during implantation period. Sherwin et al.,⁽⁷⁾ demonstrated very weak gp130 immunostaining of endometrial tissue during the secretory phase of menstrual cycle. Moreover, the cultured endometrial biopsies taken from infertile women secreted gp130 significantly less than fertile biopsies did. Depending on that the biological activity of LIF in the endometrium is affected by the levels of their soluble gp130 receptor they reported that the reduced secretion of gp130 by infertile women during the window of implantation points to a functional difference in endometrium at that time. The issue that fertile women secreted higher soluble gp130 in uterine flushing in presence of gp130 mRNA expression as low as in infertile women is not conflicting because soluble gp130 can be produced also by proteolytic cleavage of the membrane bound receptor

CONCLUSION

Endometrium of women complaining of idiopathic infertility has abnormalities in expression and secretion of important cytokines as LIF and its gp130 receptor molecules which may contribute to altered uterine receptivity and so infertility. The study also suggests that uterine flushing may help to detect the unresponsive uterus before assisted reproduction treatments and in the future, help to verify the normalization of cytokine concentrations to improve uterine receptivity before ovarian stimulation.

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