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The low expression of leukemia inhibitory factor in endometrium: Possible relevant to unexplained infertility with multiple implantation failures

Man Wu [a], Yongixang Yin [a], Min Zhao [a], Lingqing Hu [a,*], Qi Chen [a,b,*]

[a] Wuxi Maternity and Child Health Hospital, Nanjing Medical University, China
[b] Department of Obstetrics & Gynaecology, The University of Auckland, New Zealand

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ABSTRACT

Unexplained infertility affects 25% of infertile couples. Cytokines and growth factors have been suggested to play an important role in the initial process of successful implantation in humans and failures in their production may be a cause of unexplained infertility. Leukemia inhibitory factor (LIF) and Interleukin-6 (IL-6) have demonstrated their importance in implantation in both animal and human studies. Lower expression of LIF is found in proliferative phase and maximal expression is found in secretory phase of the menstrual cycle. Lower expression of LIF is also found in secretory phase endometrium in patients with infertility. However, studies investigating whether the levels of LIF in proliferative phase are associated with multiple implantation failures (MIFs) are limited. 30 Endometrial biopsies in proliferative phase from unexplained infertile women with MIF with normal hormone levels were collected. The expression of LIF, IL-6 and its receptor gp130 were measured by immunohistochemistry and western blotting. Moderate expression of LIF in the proliferative phase and high expression of LIF in the secretory phase were found in fertile women. However, lower expression of LIF was found in unexplained infertile women with MIF compared to fertile women. There was no difference in endometrial IL-6 and gp130 expression between unexplained infertile women with MIF and fertile women. LIF expression is independent of the process of embryo and dependent partially on the maternal sex hormone levels. Our data suggest that the initial lower expression of LIF in proliferative phase may be one of the causes for multiple failure of implantation.

1. Introduction

Unexplained infertility is usually diagnosed after investigation show normal male semen analysis and assessment of female ovulation, uterus and fallopian tubes [1]. Unexplained infertility is seen in up to 25–30% of infertile couples [2,3]. Although the causes of infertility are unclear, the potential causes of unexplained infertility could be implantation failure, immunological dysregulation , endometriosis or eggs from elder women [4]. The uterine environment is thought to be one major causes of reproductive failure.

Embryo implantation is the group of processes by which the developing blastocyst adheres to, and embeds into, the receptive endometrium [5]. Alternations in the expression of molecules on the endometrial epithelial cell surface have been observed during the conversion of the endometrial epithelium from a non-receptive to a receptive state [6] following exposure to 17-b-estradiol (E) followed by progesterone (P) [7]. During this stage, many endometrial derived cytokines and growth factors play an important role in the initial process of successful implantation in human. Any failure in the production or regulation of these cytokines or growth factors may be a cause of unexplained infertility.

Among the cytokines, leukemia inhibitory factor (LIF), which belongs to the Interleukin-6 (IL-6) family of cytokines and IL-6, were conclusively demonstrated its importance in animal and human studies [8]. Maternal LIF affects trophoblast growth and development and is essential for implantation and has been described as a marker of the embryo implantation process [9]. LIF is expressed on endometrium of uterus. Low levels of LIF are found in the proliferative phase and maximal expression is found during the mid-secretory phase which occurs between days 5 and 10 following the luteinising hormone (LH) surge [10]. This suggests the expression of LIF is largely independent of the embryo and is dependent at least partially on the maternal sex hormone levels [11].

IL-6 shows a similar pattern of expression to LIF in endometrial tissues. Weak staining for IL-6 in the endometrium was shown in the proliferative phase of the menstrual cycle whilst increased staining was shown in mid secretory phase in fertile women. A recent study has shown that serum levels of IL-6 were increased in

* Corresponding authors. Address: Wuxi Maternity and Child Health Hospital Affiliated Nanjing Medical University, 48 Huaishu Street, Wuxi, Jiangsu Province, China.
E-mail addresses: hlq62@hotmail.com (L. Hu), q.chen@auckland.ac.nz (Q. Chen).

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the proliferative phase of the menstrual cycle in women with unexplained infertility [12] although increased serum levels of IL-6 in early secretory phase in infertile women have also been reported [13].

Glycoprotein 130 (gp130) is a transmembrane protein which is a signalling transducer for cytokines of the IL-6 family and is expressed in the endometrial glandular epithelium [14]. Binding of LIF or IL-6 to its receptor promotes the formation of a receptor complex with gp130. The biological activity of LIF and IL-6 is affected by the levels of gp130. LIF acts on cells through binding to a heterodimeric receptor that includes LIF receptor and gp130 [15].

The levels of LIF in secretory phase in infertile women have been well investigated which suggest the reduction of LIF levels in endometrium and in uterine flashings in infertile women [16,17], although a study reported no difference of LIF levels between fertility and infertility in secretory phase [18]. In humans the expression of these cytokines seems to be important both in proliferative phase and secretory phase of the cycle because impaired endometrial development may cause infertility [19] in particular for women with multiple implantation failures (MIFs). The levels of LIF in the proliferative phase of the menstrual cycle in women with unexplained infertility and MIF have not been fully investigated. Therefore in this study we investigated the possible role of LIF signalling in the pathogenesis of unexplained infertility with MFI by studying the expression of LIF, IL-6 and its receptor gp130 in endometrium in proliferative phase.

2. Materials and methods

This study was approved by the Ethics Committee of Wuxi Maternity and Child Health Hospital of Nanjing Medical University, China. All patient-derived tissues were obtained with written informed consent.

2.1. Study population

Thirty women with unexplained infertility who were attending to Wuxi Maternity and Child Health Hospital of Nanjing Medical University, China for treatment of infertility from December 2010 to October 2012 were included into this study. All the women had history of multiple implantation failures. The median age of all patients at diagnosis was 28 years old (range from 22–46 years old). The median menstrual cycle of infertile women was 28 days (range from 26–37), and the average duration of menses was 6 days. All the women had normal ovarian function. The hormone levels including follicle stimulating hormone (FSH), luteinising hormone (LH), prolactin (PRL) and thyroid in these women were in the normal range in the time of endometrial biopsies collection.

The endometrial biopsies samples were collected during the proliferative phase (day 10 to day 14 of menstrual cycle) before any treatment and were divided into two pieces for immunohistochemistry analysis and western blotting of LIF, IL-6 and gp130. The control endometrial samples were collected from 16 fertile women who had hysterectomy due to fibroids in the proliferative phase of menstrual cycles. The median age of controls was 27.5 years old (range from 20–45 years old). All the fertile women as least had one live birth without history of infertility or miscarriage. And none of fertile women had taken any steroid hormones for at least 3 months before sample collection.

2.2. Immunohistochemistry

The expression levels of LIF, IL-6 and gp130 in endometrium were analysed by immunohistochemistry on paraffin-embedded sections. Antigen retrieval was performed by treatment with citric acid (pH 6.0) for 15 min. Non-specific antibody binding was blocked by incubating with 10% fetal calf serum for 20 min. Rabbit anti-human LIF polyclonal antibody (Epitomics USA, 1:200), mouse anti human gp130 and IL-6 monoclonal antibody (Santa Cruz Inc., and 1:100) were added for 1 h at room temperature. Sections were then washed with PBS and incubated with HRP-Polymer anti-Mouse/Rabbit IgG (Maxvision™2 kit, Maxim. BIO, China) for 15 min. The antigen–antibody complexes were visualized using DAB and counterstained with haematoxylin.

2.3. Immunohistochemical semi-quantification

15 images in each sample from each group were taken with the microscope settings unaltered. Semi-quantitative analysis of LIF, IL-6 and gp130 expression based on the combination of staining intensity of immunohistochemical images with the percentage of positive cells was performed by a published method [20]. Briefly, no staining is scored as 0; 1–10% of positive cells stained scored as 1; 11–50% as 2; 51–80% as 3; and 81–100% as 4. Staining intensity is rated on a scale of 0–3, with 0 = negative; 1 = weak; 2 = moderate, and 3 = strong. The raw data were converted by multiplying the quantity and staining intensity scores. Negative controls were stained without prior incubation with the primary antibody. Scoring the sections was done by two independent people and the score points were presented as the average.

2.4. Western blotting

Endometrial samples from five women with unexplained infertility and five women from the control group were lysed and centrifuged at 12,000 rpm for 30 min to remove cellular debris. Thirty micrograms per lane of protein from individual samples was loaded for SDS–PAGE and then transferred onto a PVDF membrane (Millipore, Billerica, MA). After blocking with 5% non-fat milk containing 0.5% Tween 20 for 1 h at room temperature the membrane was incubated with rabbit polyclonal anti-human LIF (1:1000, Epitomics USA) overnight at 4 °C. The membrane was then incubated with HRP-conjugated secondary antibodies (1:5000) for 1 h at room temperature. Protein was analysed by ECL (Perkin Elmer, Waltham, MA) with β-actin as a loading control. Western blotting was repeated with five different endometrial tissues.

2.5. Statistical analysis

The semi-quantitative immunohistochemistry data of LIF or gp130 expression was expressed as the median ± range obtained from 30 patients and 16 controls. Statistical differences between the control and unexplained infertility groups were assessed by Mann–Whitney U-test using Prism software package. Semi-quantification of immunohistochemical staining was analysed using a Mann–Whitney Test by Prism software package. A P value of <0.05 was considered to be statistically significant.

3. Results

3.1. The levels of LIF expression in the endometrium of infertile women were significantly reduced

To investigate the LIF expression during the menstrual cycle, we measured the expression of LIF in the endometrium during the proliferative phase and secretory phase. The expression of LIF was moderate in the glandular epithelial cells in proliferative phase compared to that in secretory phase in fertile women (Fig. 1A and B). However, the expression of LIF in proliferative phase in unexplained infertile women with MIF was lower