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Endometrial CXCL13 Expression Is Cycle Regulated in Humans and Aberrantly Expressed in Humans and Rhesus Macaques With Endometriosis

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Abstract

C-X-C ligand 13 (CXCL13), a regulator of mucosal immunity, is secreted by human endometrial epithelium and may be involved in embryo implantation. However, cyclic expression of human endometrial CXCL13 in health and disease is not well studied. This study examines cycle stage-specific endometrial CXCL13 expression in normal humans when compared to those with biopsy-confirmed, stage 1 to 4 endometriosis using real-time reverse transcriptase, real-time polymerase chain reaction and immunohistochemistry. Eutopic endometrial CXCL13 expression was also compared between normal, control Rhesus macaques, and macaques with advanced endometriosis. In healthy women, CXLC13 messenger RNA expression was minimal in the proliferative phase and maximal in the secretory phase. However, in the presence of endometriosis, proliferative-phase endometrial expression markedly increased in both humans and rhesus subjects ($P < .05$). The cross-species and cross-stage concordance suggests a pathophysiologic role for CXCL13 in endometriosis and its use as a biomarker for disease.

Keywords

CXCL13, endometriosis, proliferative phase

Introduction

Endometriosis is a common, complex disease affecting about 5% to 10% of reproductive-age women and contributes to chronic pelvic pain and infertility.[1-4] Defined by the presence of endometrial glands and stroma at extrauterine sites, endometriosis appears to alter the eutopic endometrial phenotype, with the endometrium showing changes in molecular markers for inflammation[5,6] and steroid metabolism.[7] Such eutopic endometrial changes have been suggested as potential diagnostic biomarkers for endometriosis.[8-10] However, these markers have, to date, been insufficiently sensitive and specific for broad clinical use. Imaging has also been proposed as a diagnostic tool, but it also lacks sufficient sensitivity.[11]

Although a diagnosis of endometriosis may be suggested by patient history, symptoms, and physical signs, clinical presentation is variable and nonspecific. Given the shortcomings of biomarkers, imaging, and clinical examination, surgical visualization (with histologic confirmation) remains the only sensitive and specific diagnostic test for endometriosis.[12-15]

The pathophysiology of endometriosis is still debated. Retrograde menstruation and subsequent tissue persistence and proliferation are a commonly accepted theory; however, the incidence of retrograde menstruation is similar in women with and without endometriosis.[16] It is proposed that women who develop endometriosis lesions have differences in the endometrial regulation of tissue proliferation, tissue remodeling, inflammation, and other immune processes.[17-22] Additionally, it has been hypothesized that endometriosis-related pain and

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infertility results from an endometrial inflammatory response, chemokine-mediated immune cell recruitment, and subsequent chronic activation of the immune system.[23,24]

Cytokines and chemokines play critical roles in immune system activation and function but have also been implicated in the upregulation of adhesion molecules that promote cancer invasion, in a manner analogous to that proposed for endometriosis lesions.[25,26] Furthermore, these inflammatory mediators may direct alterations in immune surveillance that facilitates implantation and survival of ectopic endometrial tissues.[27,28] Thus, chemokines may play a functional role in the pathophysiology of endometriosis and therefore may be a pertinent target for development of novel diagnostic and therapeutic approaches.

In this article, the experiments are focused on expression of a single chemokine, C-X-C ligand 13 (CXCL13). Possible endometrial functions of CXCL13 include regulation of both immunity and reproductive functions.[29,30] In the pleural and peritoneal cavities, CXCL13 is constitutively expressed by macrophages and cells of nonhematopoietic origin. At these sites, it is an important promoter of natural antibody production and local immunity via specific chemoattraction of the B-1 subset of B cells,[29] and mice homozygous for CXCL13 deletion have a severe reduction in peritoneal and pleural B-1 cells.[31] Interestingly, CXCL13 is also abundantly secreted by human endometrial epithelium and is specifically taken up by embryos with high implantation potential.[32] Endometrial expression has been shown to be elevated during the window of implantation in women undergoing *in vitro fertilization* (IVF) versus those in natural menstrual cycles.[33] Additionally, microarray data are suggestive of an increase in CXCL13 in the secretory phase,[34] although microarray data are not consistent.[35]

With the aforementioned studies suggesting the importance of CXCL13 in immunity and reproductive functions, we sought to examine how endometriosis altered CXCL13 in women with this disease. In this study, we examined the expression of the CXCL13 chemokine by both reverse transcriptase, real-time polymerase chain reaction (RT-PCR) and immunohistochemistry in endometrium across the menstrual cycle in normal, reproductive-age women and then compared these findings to expression in the endometrium of women collected prior to surgical confirmation of endometriosis. The proliferative and secretory phases were analyzed separately due to recent evidence suggesting endometrial alterations in both phases in women with endometriosis.[36,37] Since factors altered in endometriosis in multiple species are more likely to play a fundamental pathophysiological role in disease, we also present data on CXCL13 expression in eutopic endometrium of Rhesus macaques with and without naturally occurring endometriosis.

Materials and Methods

Participants

Study population and endometrial sampling. Participants signed an informed consent for this institutional review board (IRB)-approved protocol for office biopsy on normal women timed to luteinizing hormone (LH) surge. Normal controls (n = 50) were recruited and included healthy volunteers between the ages of 18 and 38 who had regular, cyclic menses. Regular menses was defined as a cycle length of 24 to 35 days. None had signs or symptoms of endometriosis or fertility problems, defined by unprotected intercourse for greater than 1 year without conception. Participants had been off of hormonal therapy for a minimum of 3 months prior to biopsy. This clinical information was obtained via a standardized questionnaire that was reviewed by the study coordinator and principle investigator. They did not undergo laparoscopy, but based upon prevalence data, approximately 5% to 10% (3-5 of 50) of healthy women would have undetected endometriosis to skew the findings away from significance.[38] All normal controls were sampled only once.

Patients with endometriosis (n = 27) came from a population who originally carried the diagnosis of unexplained infertility between the ages of 21.4 and 39.2. They were found to have stage 1 to 4 endometriosis as confirmed via laparoscopic inspection and pathologic diagnosis. Patients with endometriosis had been off hormonal therapy for a minimum of 3 months prior to biopsy. Cycle timing in all women was determined relative to urinary LH rise. Endometrium was obtained by endometrial suction curettage (3 mm flexible plastic instrument) from controls and patients with endometriosis. Portions of endometrial biopsies were frozen in liquid nitrogen in the clinic and transported to the laboratory where they were stored at −80°C until further use, while the rest was fixed in 10% buffered formalin for paraffin embedding and sectioning. All tissues were obtained in accordance with the Committee for the Protection of Human Subjects at the University of North Carolina and Greenville Hospital System under approved IRB protocols.

Messenger RNA expression. Expression of human endometrial CXCL13 messenger RNA (mRNA) was determined using quantitative real-time RT-PCR (qRT-PCR) analysis. The qRT-PCR was performed on an MX3000 real-time thermocycler (Stratagene, La Jolla, California) with the following conditions: total RNA was extracted from frozen endometrial biopsies using Trizol Reagent (Ambion, Life Technologies, Grand Island, New York) according to the manufacturer's suggested conditions. RNA quantification was performed using RiboGreen (Invitrogen; Carlsbad, California) with a ribosomal RNA standard curve. First strand cDNA was synthesized using AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, Santa Clara, California) according to the manufacturer's specifications.

Relative quantitation of specific mRNA species was achieved by real-time PCR using predesigned Taqman probe and primer sets (Applied Biosystems Inc, Foster City, California), and relative expression was calculated using the ΔΔCt method. The PCR primers and fluorogenic probes included Hs04194521-s1 (peptidylprolyl isomerase A [PPIA]) and Hs00757930-m1 (CXCL13; Applied Biosystems Inc). The PPIA (cyclophilin A) gene was used as a constitutive housekeeping control since previous work suggested that PPIA

exhibits little variation across the menstrual cycle and disease states (Steven L. Young, MD, PhD, unpublished data, 2014)—indeed, we saw little variation in Ct for PPIA across the samples. Probe-primer sets were designed across exon–exon junctions to prevent detection of genomic DNA. The data were grouped by cycle phase and analyzed by Kruskal-Wallis nonparametric testing for samples across the cycle and Dunn Multiple comparison test when comparing endometriosis and control samples.

Immunohistochemistry. Immunohistochemistry for CXCL13 was performed on paraffin sections of endometrium from women with endometriosis and normal controls. All reactions were performed at room temperature unless noted otherwise. Tissue sections were deparaffinized in toluene, rehydrated through descending ethanols, rinsed in phosphate-buffered saline (PBS), and pretreated with 5% hydrogen peroxide in 83% methanol for 30 minutes to block endogenous peroxidases. Slides were incubated with normal rabbit serum (ABC-kit, Vector Labs, Burlingame, California) for 30 minutes to block nonspecific immunoglobulin (Ig) G reactions. Sections were then incubated at 4°C overnight in a humidity chamber with a goat polyclonal antibody raised to detect CXCL13 (R&D Systems; Minneapolis, Minnesota); primary antibody was omitted from slides used as negative controls.

After overnight incubation with primary antibody, slides were rinsed in PBS, incubated in normal rabbit serum for 10 minutes, and incubated with the 1:200 biotinylated rabbit anti-goat IgG (Vector Labs) for 60 minutes. The slides were rinsed with PBS and reacted with 1:400 avidin DH–biotinylated horseradish peroxidase H complex for 60 minutes and rinsed in PBS. The slides were incubated for 10 minutes in diaminobenzidine peroxidase substrate (Vector Labs) to visualize antibody–antigen complexes. Sections were then stabilized by incubating the slides in double-distilled water for 5 minutes. Slides were counterstained with hematoxylin, dehydrated in ascending ethanols, cleared through a series of xylene, and then sealed under Permount. Images were taken with an SPOT Insight 4 camera using a Nikon E600 Eclipse microscope (Melville, New York).

Macaque Subjects

Study population and endometrial sampling. In the initial phase, endometrium was obtained from Rhesus macaques (*Macaca mulatta*) with naturally occurring, advanced endometriosis (similar to human stage 4; n = 11) and normal controls (n = 7). These were obtained from previous studies conducted at the Oregon National Primate Research Center (ONPRC).[39,40] Animal care was provided by the veterinary staff of the ONPRC Division of Comparative Medicine after review and approval by the ONPRC Animal Care and Use Committee. To ensure endometriosis-free control samples, animals were ovariectomized and treated with Silastic capsules that release 17β-estradiol (E_2; Sigma, St. Louis, Missouri) and E_2 plus progesterone to produce artificial menstrual cycles as described.[39,40] These implants produced 109.7 ± 8.9 pg E_2 and 4.27 ± 0.37 ng progesterone /mL in serum in the macaques.

Subsequently, we studied normal controls (n = 11) and induced endometriosis models (n = 14) across the menstrual cycle utilizing artificial cycles. This induced model has been previously described in detail.[39,40] In order to study proliferative endometrium, endometrial tissues were collected via hysterectomy specimens from artificially cycled macaques treated with E_2 for 5 to 7 days following progesterone withdrawal. In order to study secretory endometrium, endometrial tissues were collected via hysterectomy specimens from artificially cycled macaques treated with E_2 plus progesterone for 14 days.[39,40]

Animals with advanced endometriosis were diagnosed during physical examination, and this diagnosis was confirmed by conventional ultrasound, to identify sonographic features of endometriomas, and needle biopsy, with aspiration of red- and/or chocolate-colored fluids. Finally, all subjects had histologic confirmation once surgical pathology was obtained at the time of tissue procurement. Endometrium was collected by dissection from the uterus removed from the animals either during the proliferative phase (mean E_2 = 145.5 ± 16.3 pg/mL) or during the secretory phase (mean progesterone = 3.13 ± 0.21 ng/mL).

For both control and endometriotic animals, the uterus was dissected free of connective tissue adhesions and endometriotic tissue, bisected along the longitudinal axis from the fundus to cervix, and then further divided into quarters. Full-thickness tissue sections (~5 mm) extending from the uterine lumen to the outer myometrium were taken from each quarter. Tissue blocks were fixed in 4% paraformaldehyde and subsequently embedded in paraffin. The remaining endometrium was separated from the outer myometrium with iris scissors, weighed, and frozen separately in liquid nitrogen for subsequent mRNA analysis.

Messenger RNA expression. Frozen samples of endometrial tissue were thawed in 10 volumes of TRIzol (Invitrogen), and total RNA was isolated, purified, and quality assessed as described.[41] Briefly, total RNA (1 μg) was reverse transcribed with random hexamer primers (Promega Corporation, Madison, Wisconsin) using an Omniscript RT kit (Qiagen, Valencia, California). Transcript levels in each sample were quantified against a 5-point standard curve, derived from pooled cDNA from all samples, prepared by 1:10 serial dilution. The relative transcript levels of CXCL13 were normalized to the expression of S10. Each reaction contained TaqMan probe specific for each gene of interest and was performed in MicroAmp Fast Optical 96-well plates on an ABI 7500 Fast Real-time PCR System. Real-time PCR reagents and TaqMan probes were purchased from Applied Biosystems Inc. Primer and probe were designed using Primer Express software (Applied Biosystems Inc) and are as follows: CXCL13—forward, 5′-TGTGTCCAAGAGAGCTCAGTCTTT-3′, reverse, 5′-GGCGAGATTTGAATT CGATCA-3′, probe, 5′-TCCCCAGACGCTTCA-3′; S10—forward, 5′-AATGTGCCC AACCTTCATGTC-3′, reverse, 5′-TCCAGG CAAACTGTTCCTTCA-3′, probe, 5′-

TGAAGGCCATGCAGTCTCTCAAGTCCC-3′. Data were analyzed by Mann-Whitney *U* test, $P < .05$.

Immunohistochemistry. Localization of CXCL13 protein was conducted on paraffin-embedded samples. Serial 5-μm sections were cut and mounted on Superfrost Plus slides (Fisher Scientific, Waltham, Massachusetts), deparaffinized in xylene, and rehydrated stepwise in descending ethanol. Antigen retrieval was performed by heating the sections in citrate buffer (pH 6.0) for 10 minutes in a pressure cooker. All steps were performed at room temperature (~23°C) unless noted otherwise. Slides were rinsed in PBS, treated with 3% hydrogen peroxide (in methanol) for 30 minutes to block endogenous peroxidases, then incubated with normal serum (ABC-kit, Vector Labs) for 20 minutes to block nonspecific IgG reactions. Sections were then incubated at 4°C overnight with a goat polyclonal antibody raised to detect CXCL13 (R&D Systems); primary antibody was omitted from slides used as negative controls.

After overnight incubation with primary antibody, all slides were rinsed in PBS (containing 0.075% nonionic detergent BRIJ), incubated with normal serum for 20 minutes, and then incubated with the biotinylated rabbit anti-goat IgG (Vector Labs) for 30 minutes. The slides were rinsed with PBS, reacted with avidin–biotin peroxidase reagents (for 60 minutes), and rinsed in Tris buffer (pH 7.6). The slides were incubated in 0.025% diaminobenzidine to visualize antibody–antigen complexes. Sections were then stabilized by incubating the slides in 0.025% osmium tetroxide (for ~1 minute). Sections were counterstained with hematoxylin, dehydrated in ascending ethanols, cleared through a series of xylene, and then sealed under Permount.

Slides were viewed on a Zeiss AxioImager A.1 microscope (Carl Zeiss, Inc., Oberkochen, Germany) with planapochromatic lenses. Digital photomicrographs were captured with a Leica DFC 480 camera (Leica, Wetzlar, Germany). All photomicrographic plates were background adjusted, cropped, and subsequently annotated in Photoshop Creative Suite 4 (Adobe Systems, Seattle, Washington).

Ethical approval. Participants signed an informed consent for this IRB-approved protocol. Animal care was provided by the veterinary staff of the ONPRC Division of Comparative Medicine after review and approval by the ONPRC Animal Care and Use Committee.

Results

Participants

To examine the changes in the expression levels of CXCL13 across the menstrual cycle, endometrial biopsies were taken from normal, reproductive-age women in proliferative (P; n = 17), early secretory (ES; n = 8), mid-secretory (MS; n = 16), and late-secretory (LS; n = 9) phases. The early/mid/late portions of the secretory phase are defined as follows: early LH +2-5, mid LH +6-10, and late LH +11-14. No patients were menstruating at the time of biopsy. Average participants age was 25.5 years old (range = 18-34 years old).

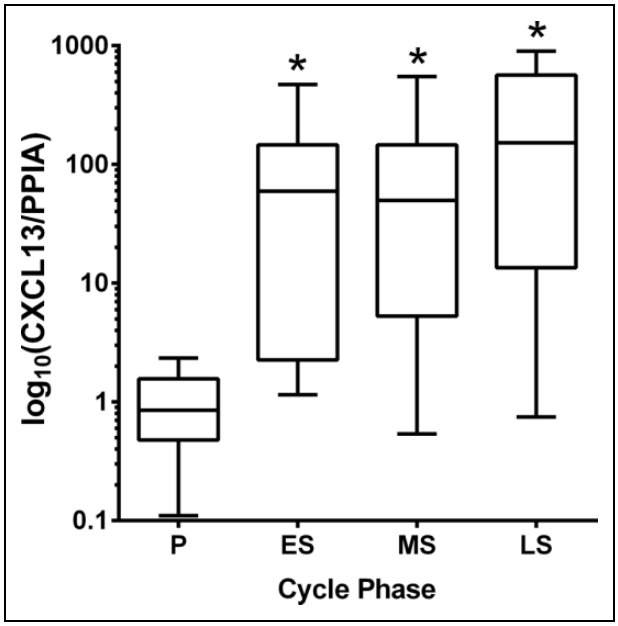

Figure 1. C-X-C ligand 13 (CXCL13) gene expression increases during the secretory phase of the normal human menstrual cycle. Relative expression levels of CXCL13 messenger RNA (mRNA) shown by real-time polymerase chain reaction (PCR) in controls (n = 50) across the menstrual cycle. P, proliferative (n = 17); ES, early secretory (n = 8); MS, mid-secretory (n = 16); LS, late secretory (n = 9). Median lines are shown and box incorporates 75% of the samples. * indicates $P < .001$ by Kruskal-Wallis test compared to P.

In these normal participants, CXCL13 expression was highly cycle regulated, with median mid-secretory mRNA expression nearly 60-fold greater than that seen in the proliferative phase ($P < .001$; Figure 1). Immunohistochemistry confirmed that protein expression mirrors the changes seen in mRNA expression and localizes expression to both epithelium and stroma (Figure 2). These findings strongly suggest that changes in mRNA expression translate to changes in protein abundance and that both epithelial and stromal CXCL13 expressions may be directly or indirectly stimulated by progesterone.

Given the cycle variation between the proliferative phase and the secretory phase, we compared CXCL13 expression in the eutopic endometrium of healthy participants (n = 50) to that of women with endometriosis (n = 27; Figure 3). The patients with endometriosis had a mean age of 31.6 years (range = 21.4-39.2 years). All came from a prospectively obtained population with unexplained infertility (normal male partner, ovulatory cycles, and at least 1 patent fallopian tube).

Using the control proliferative phase samples as a reference (C Proliferative), we compared this to proliferative phase samples from women with endometriosis (Endo Proliferative) and find that CXCL13 is significantly increased. Upon examining the secretory samples, we find that both control (C) and endometriosis (Endo) samples display increased levels of CXCL13

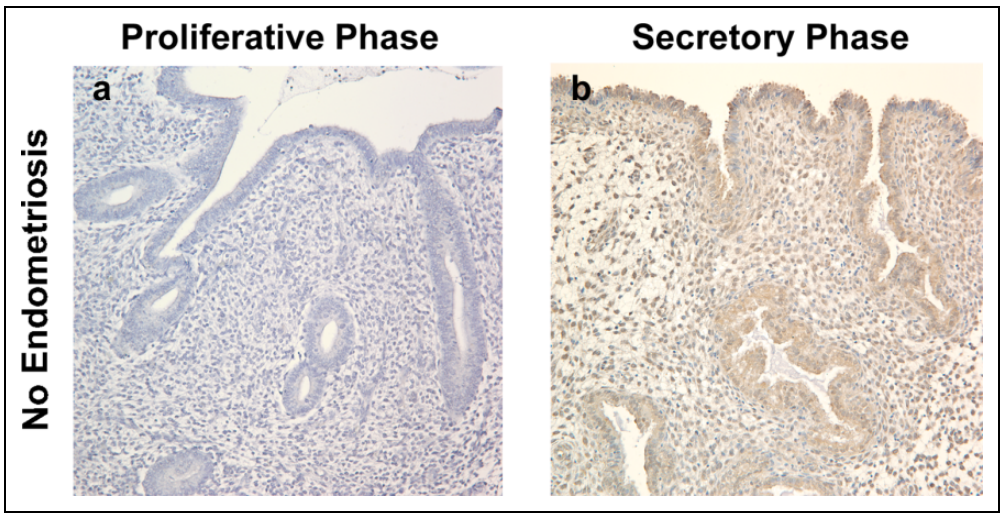

Figure 2. C-X-C ligand 13 (CXCL13) protein is expressed in the glandular and stromal compartments of normal human endometrium. Representative photomicrographs demonstrating CXCL13 expression in endometrial biopsies from normal, reproductive-age women in the proliferative phase (A) and the secretory phase (B). CXCL13 immunohistochemically stains brown and the nuclei are counterstained blue with hematoxylin. Representative photomicrographs of human endometrium are shown at 200× magnification. (The color version of this article is available at http://rs.sagepub.com.)

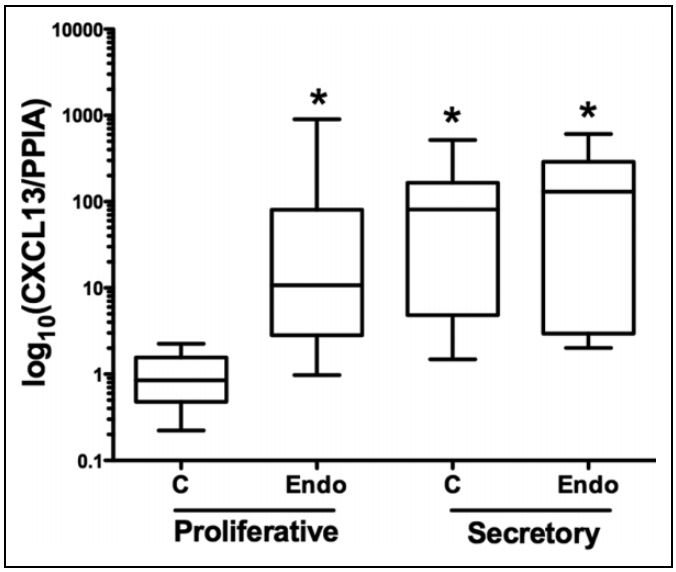

Figure 3. C-X-C ligand 13 (CXCL13) gene expression increases aberrantly during the proliferative phase of the menstrual cycle in women with endometriosis. CXCL13 messenger RNA (mRNA) expression from control proliferative (C Proliferative) and control secretory (C Secretory). Endometriosis proliferative (Endo Proliferative) and endometriosis secretory (Endo Secretory) phases of the menstrual cycle were compared from patients with endometriosis (Endo) and control endometriosis-free, reproductive-age controls (C) shown by real-time PCR. Median lines are shown and box incorporates 75% of the samples. * indicates $P < .001$ by Dunn Multiple Comparison test comparing control samples in the control proliferative phase to other groups. The maximum level seen in the control proliferative samples was below the lowest 25th percentile in patients with endometriosis. Control proliferative (C Proliferative; n = 17); endometriosis proliferative (Endo Proliferative; n = 12); control secretory (C Secretory; n = 33); endometriosis secretory (Endo Secretory; n = 15).

with no additional increase seen in endosecretory samples. In the proliferative phase, the controls demonstrated a consistent, low level of CXCL13 expression, while the samples from patients with endometriosis exhibited a wide range of expression levels, with a 10-fold median increase and a greater than 1000-fold maximal increase. Importantly, the maximum level seen in the controls was less than the 25th percentile in patients with endometriosis in the proliferative phase.

Immunolocalization of CXCL13 in the proliferative endometrium of participants without endometriosis displayed minimal expression of CXCL13 protein that mirrored the quantitative mRNA data. Immunolocalization of CXCL13 in the proliferative endometrium of patients with endometriosis showed expression primarily in the glandular and luminal epithelium with intensities proportional to changes seen in mRNA levels (Figure 4). These findings demonstrate that many women with endometriosis have increased epithelial CXCL13 expression in the proliferative phase.

Rhesus Macaque Subjects

Given the significant findings in human endometrium, we next examined endometrial samples from healthy Rhesus macaques and compared them to samples of Rhesus macaques with concomitant naturally occurring endometriosis to examine cross-species concordance. We initially examined endometrial tissue from Rhesus macaques with (n = 7) and without (n = 11) endometriosis. In the controls, the mean age was 9.8 years(range = 6.6-12.1 years) and in the naturally occurring endometriosis the mean age was 13.9 years (range = 10.22-18.10 years). The mRNA data from macaques mirrored that from humans in that there was a significant increase in overall expression of CXCL13 in the endometrium of macaques with endometriosis (Figure 5).

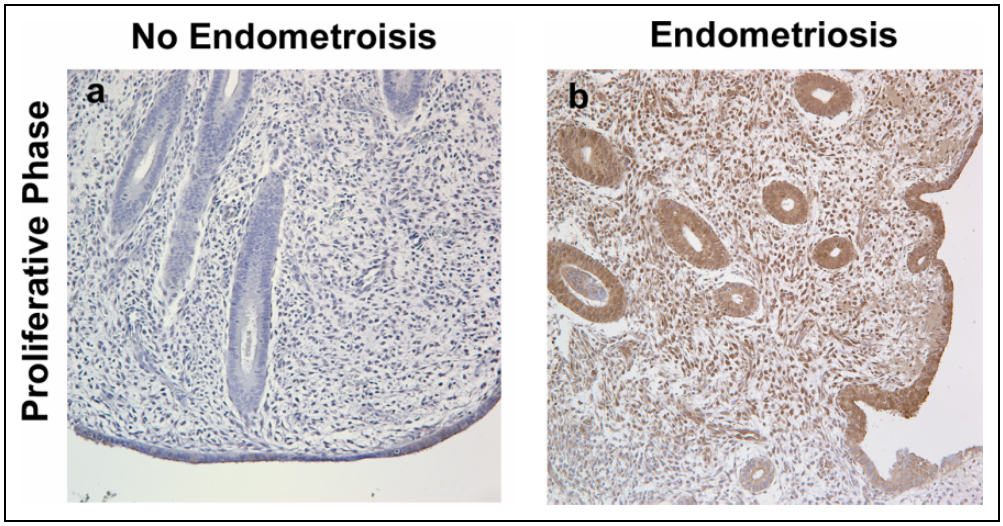

Figure 4. C-X-C ligand 13 (CXCL13) protein is expressed in the glandular and stromal compartments of proliferative phase endometrium in women with endometriosis. Representative photomicrographs demonstrating CXCL13 expression in normal (A) and in endometriosis (B) in reproductive-age women in the proliferative phase. CXCL13 immunohistochemically stains brown and the nuclei are counterstained blue with hematoxylin. Representative photomicrographs of human endometrium are shown at 200× magnification.

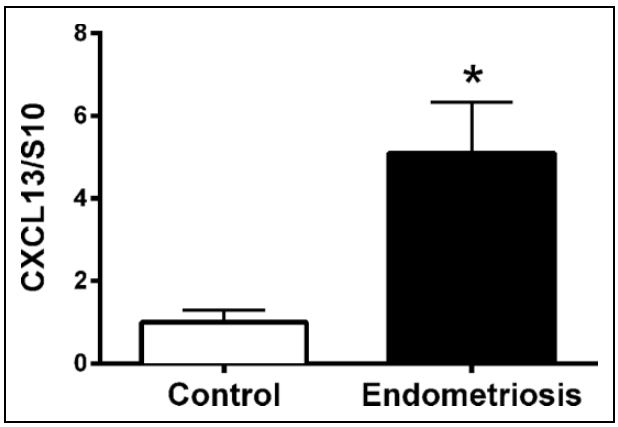

Figure 5. C-X-C ligand 13 (CXCL13) gene expression increases in the endometrium of Rhesus macaques with naturally occurring endometriosis. Endometrium from macaques with endometriosis and those without was analyzed for CXCL13 messenger RNA (mRNA) by real-time polymerase chain reaction (PCR). * indicates $P < .05$ by Mann-Whitney *U* test.

To then more closely examine at what stage the CXCL13 was aberrantly expressed in macaques with endometriosis, samples from proliferative and secretory groups were examined in subjects with surgically induced endometriosis. Samples analyzed were from the proliferative phase (n = 3) and secretory phase (n = 9) and in the endometriosis-free control animals and the proliferative phase (n = 6) and secretory phase (n = 8) from the induced endometriosis group. The induced endometriosis group was more closely age matched with the control group and had a mean age of 10.4 years (range = 7.7-12.8 years). However, we did not see the cycle-related differences noted in human endometrium (Figure 6A). Although macaques with endometriosis demonstrated a 2.7-fold mean increase in CXCL13 mRNA in proliferative phase samples when compared to proliferative phase controls (Figure 6A), this apparent change did not reach statistical significance, most likely due to a limited number of subjects in the proliferative control group. However, a more consistent and somewhat higher (3-fold) significant increase was seen in the late secretory macaque samples with endometriosis when compared to controls (Figure 6A). Immunohistochemistry for CXCL13 confirmed expression of protein in the glandular and luminal epithelium, which mirrored the increased mRNA levels by quantitative real-time PCR (Figure 6B). Thus, cycle-phase effects on normal macaque endometrial expression of CXCL13 were not detected, but importantly, endometriosis was correlated with increased CXCL13 expression.

Discussion

In this study, we analyzed the changes in human endometrial CXCL13 expression across the normal menstrual cycle, demonstrating maximal expression in the secretory phase and minimal expression in the proliferative phase. Mid-secretory phase endometrial CXCL13 expression in women with endometriosis versus controls was similar, while proliferative phase expression in women with endometriosis was dramatically elevated. Indeed, the maximum level seen in the controls was less than the 25th percentile in women with endometriosis. To our knowledge, this is the first account demonstrating upregulation of CXCL13 expression in the secretory phase of the normal, human menstrual cycle and, the first demonstration of increased expression of CXCL13 in the endometrium of humans and macaque subjects with endometriosis. The cross-species data concordance strongly supports the hypothesis that endometrial CXCL13 expression plays an important role in the pathophysiology of the disease.

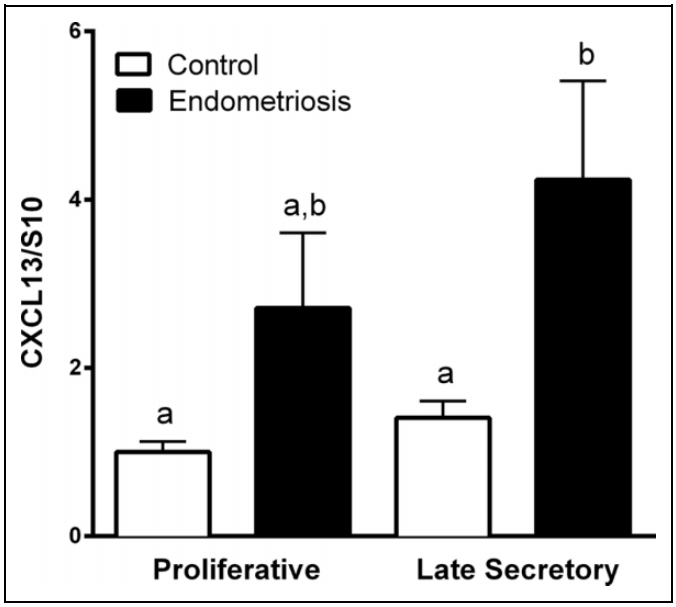

Figure 6. C-X-C ligand 13 (CXCL13) increases in Rhesus macaques with endometriosis in the proliferative and secretory phases of the menstrual cycle. A, CXCL13 messenger RNA (mRNA) expression was analyzed in the proliferative phase (n = 3) and secretory phase (n = 9) and from endometriosis-free control animals in the proliferative phase (n = 6) and secretory phase (n = 8). Bars marked "a" are $P < .05$ versus those marked "b" by analysis of variance (ANOVA) and Neumann-Keuls post hoc testing. B, Representative photomicrographs demonstrating immunohistochemical staining for CXCL13 in the proliferative phase and secretory phase of the menstrual cycle in Rhesus macaques. CXCL13 staining is brown and the nuclei are counterstained blue with hematoxylin. Insets are negative controls with primary antibody omitted.

In contrast, the microarray data in Burney et al showed that CXCL13 expression was reduced in women with endometriosis, but those findings were confined to the secretory phase.[35] The reasons for these differences are unclear, but given the greater precision of PCR versus microarray and the general concordance between immunohistochemistry and PCR, we believe that the data in our report are likely more generalizable.

The largest fold changes between patients with endometriosis and controls were seen in the proliferative phase human samples. The finding of proliferative phase changes is interesting in that most studies have examined the secretory phase.[42] However, a few recent articles have suggested a proliferative phase abnormality in women with endometriosis.[36,43] Specifically, Bromer et al introduced sonographic evidence of alterations in proliferative phase endometrial thickness supporting a ''proliferative phase defect'' in patients with endometriosis and other specific causes of infertility.[36] Petracco et al found proliferative phase expression of specific microRNA species altered in endometriosis.[43] Our findings lend further molecular and histologic evidence that an endometrial abnormality in women with endometriosis can be seen in the proliferative phase, a time when abnormalities are independent of an altered response to progesterone.[35,44,45]

The specific role that CXCL13 might play in the endometriosis disease processes is unclear, but knowledge of CXCL13 effects in other systems may be instructive. C-X-C ligand 13 is a chemokine that is strongly expressed in the follicles of the spleen, lymph nodes, and Peyer patches and preferentially promotes the migration of B lymphocytes (especially B-1 cells) when compared to T lymphocytes and macrophages.[46] Indeed, CXCL13 plays an important role in migratory synergism for homeostatic responses and a pronounced role in inflammatory trafficking of B cells.[47-49] In addition, elevated CXCL13 production has also been shown to be correlated with autoimmune disease.[50-56] These findings may be directly relevant to endometriosis, given the growing body of data detailing the intimate association between the immune system and endometriosis pathophysiology.[57-60] Thus, CXCL13 misexpression adds to our understanding of this complex interplay between the immune system, endometriosis, and infertility.

C-X-C ligand 13 also plays a role in cancer adhesion and invasion, which might be considered an analogous process to the endometrial tissue adherence and invasion into the peritoneum in women with endometriosis. In prostate cancer, CXCL13 induces cell adhesion and invasion in a CXCR5-dependent manner.[61,62] In breast cancer, CXCL13 is overexpressed with elevated levels found in the serum of patients with metastatic disease.[63] Additionally, CXCL13 can play a role in cellular trafficking. Neural precursor cells are able to cross the brain endothelium in response to interleukin 8 and CXCL13 and play a role in vascular inflammation and leukocyte recruitment.[64,65] Thus, CXCL13 might play a chemotactic role for the localization, adherence, and/or invasion of endometriosis lesions.

Finally, CXCL13's role in cell migration and tissue invasion has implications in successful embryo implantation. Our data indicate that CXCL13 expression in the human is cycle regulated and increased in the secretory phase, including the window of implantation. The interplay between CXCL13 and the embryo has been partially characterized in that CXCL13 is both secreted by human endometrial epithelium and specifically taken up by embryos with high implantation potential.[32] However, it is unclear whether the blastocyst internalizes CXCL13 in a receptor-mediated process or is simply metabolized. Thus, the hypothesis that CXCL13 plays a chemotactic role in trophoblast invasion is attractive but requires significantly more investigation.

In summary, the potential roles for CXCL13 in normal and abnormal reproductive processes are many and include embryo implantation, embryo selection during IVF cycles, endometriosis pathophysiology, and endometriosis diagnosis. However, further investigations are needed to more clearly elucidate CXCL13's specific role in these processes. A murine model may allow for detailed mechanistic studies of endometriosis development and allow testing of therapeutic drugs targeted to the CXCL13 signaling pathway. One exciting possibility suggested by the definitive dysregulation of CXCL13 in the proliferative phase of many patients with endometriosis is that CXCL13 could serve as a specific diagnostic marker. Further studies are being undertaken by our group to test the clinical usefulness of measuring CXCL13 expression in endometrial

biopsy material as well as in a less invasive cervical swab, given the secretion of CXCL13 protein by luminal epithelium.

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Authors' Note

Jason M. Franasiak and Katherine A. Burns contributed equally and should both be regarded as joint first authors. JMF and KAB were primary writers of the manuscript. JMF and LY generated data. OS, BAL, and SLY collected endometrial samples. All authors were actively involved in study design, data analysis, interpretation, discussion, and critical manuscript review. This research was completed jointly at the University of North Carolina, Greenville Hospital System, and the Oregon National Primate Research Center.

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Declaration of Conflicting Interests

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