Temporal analysis of connexin43 protein and gene expression throughout the menstrual cycle in human endometrium

Irit Granot, Ph.D.,* Nava Dekel, Ph.D.,[†] Edna Bechor, Ph.D.,*
Ilana Segal, B.Sc.,* Sheila Fieldust, B.A.,* and Amihai Barash, M.D.*

Kaplan Hospital; and The Weizmann Institute of Science, Rehovot, Israel

Objective: To analyze the pattern of connexin43 gene and protein expression in human endometrium throughout the menstrual cycle.

Design: Controlled clinical study.

Setting: An academic research center.

Patient(s): Women with 28-day menstrual cycles who had mechanical infertility and failed to conceive after IVF treatment.

Intervention(s): Endometrial and blood samples were collected on days 8, 12, 14, 21, and 25 of spontaneous menstrual cycles.

Main Outcome Measure(s): Endometrial expression of connexin43 protein and messenger RNA, endometrial thickness, and serum concentrations of gonadotropins and steroids.

Result(s): The expression of connexin43 gene and protein decreased on day 12 and day 14 of the menstrual cycle and then increased on day 21 and day 25, respectively. A serum LH surge accompanied by a peak in the FSH concentration was observed on days 12–14. The progesterone concentration increased on days 21–25, but there was no significant change in the E_2 concentration. The thickness of the endometrium increased between days 8 and 12 and did not change further between days 21 and 25.

Conclusion(s): The expression of connexin43 gene and protein in human endometrium changes during the menstrual cycle in a pattern that is associated with the secretion of LH, FSH, and progesterone. This pattern may serve as a marker for implantation competence. (Fertil Steril® 2000;73:381–6. ©2000 by American Society for Reproductive Medicine.)

Key Words: Human endometrium, connexin43, menstrual cycle

Implantation is a multistage process that involves apposition and adhesion of the blastocyst to the endometrium followed by invasion of the trophoblast between its epithelial cells. The development of a receptive endometrium is therefore necessary for successful implantation. The developmental changes that occur in the endometrium during the preimplantation period are controlled by the ovarian steroids estrogen and progesterone. In addition to their effect on the proliferation and differentiation of the endometrial cells, these hormones induce the secretion of cytokines that in turn affect the development of the embryo (1–3).

It has been suggested that in the rat, gap junctions between the epithelial cells and between the epithelial cells and the trophoblast participate in regulating embryonal invasion (4). Gap junctions are intercellular channels in closely opposed membranes of neighboring cells that mediate cell-to-cell communication (5, 6). Gap junctions consist of connexins (Cx), a growing family of highly homologous proteins that have been identified in different tissues. Three different Cx, Cx26, Cx32, and Cx43, were detected in the endometrium of rodents, rabbits, and humans.

Previous studies demonstrated that the expression of these Cx in the endometrium changes throughout the reproductive cycle (4, 7, 8–10). Further, the regulation of these changes by steroid hormones has been suggested (11, 12). These studies demonstrated that the expression of Cx26 and Cx43 genes in

rat endometrium increases at proestrus under the influence of estrogen and is reduced at estrus in response to progesterone. It has been suggested therefore that these estrous cycle–associated patterns of expression of the Cx in the endometrium point to a physiologic role of these proteins in the implantation process (4, 12, 13).

Implantation is the rate-limiting step in the success of IVF treatment. Hence, intensive attempts are being made to define biologic markers that can be used to predict implantation competence. In humans, most of these studies have analyzed physical changes in the endometrium as identified by sonography or by light or electron microscopy (3, 14, 15). However, the changes in endometrial thickness demonstrated in these studies could not be used to predict endometrial receptivity. Other studies have been directed at establishing biochemical markers such as cytokines or adhesion molecules that are secreted by the endometrium in response to its interaction with the blastocyst (16). Along this line, differences in growth factor expression between fertile and infertile groups of patients recently have been reported. However, the significance of these findings is still unclear (3).

Our study was performed in an attempt to determine whether there is a characteristic pattern of Cx43 expression in human endometrium throughout the menstrual cycle. If such a pattern were defined, it might be used to predict uterine receptivity. Spatial and temporal analyses of Cx in human endometrium have been described previously (8, 10). However, these studies were performed in women with uterine pathology who were undergoing hysterectomy. Moreover, the endometrium in each such patient was subjected to a single analysis performed at a nonspecified stage of the menstrual cycle.

We present herein the first report of a temporal analysis of Cx43 gene and protein expression in human endometrium throughout the menstrual cycle in healthy women with normal menstrual cycles. Further, in each woman, samples were collected at four specific times during the menstrual cycle. Blood samples also were collected from each patient at the same times for determination of hormone levels. We found that the different patients exhibited a similar temporal pattern of Cx43 expression. We also demonstrated a correlation between Cx43 expression and serum concentrations of gonadotropins and steroid hormones.

MATERIALS AND METHODS

Sample Collection

Eight women with 28-day menstrual cycles who had mechanical infertility and failed to conceive after IVF treatment were selected for the study. All the patients provided informed consent. On days 8, 12, 14, and 21 of a spontaneous cycle, the thickness of the endometrium was recorded by sonography, samples of the endometrium were collected with a Pipelle catheter (Prodimed, Neuilly-en-Thelle, France), and samples of blood were collected for measurement of serum concentrations of gonadotropins and ovarian steroid hormones. In four additional patients, samples also were obtained on day 25 of the cycle.

This study was approved by the Helsinki committee of the Kaplan Medical Center.

Determination of Serum Hormone Concentrations

Serum progesterone and E_2 concentrations were determined by chemiluminescent immunoassay using the Access Immunoassay System (Sanofi Diagnostic and Pasteur, Paris, France) and Immulite kits (Diagnostic Products Corporation, Los Angeles, CA), respectively. The assays were performed according to the manufacturer's protocols.

Serum LH and FSH concentrations were determined by ELISA using Enzymun-Test kits (Boehringer Mannheim, Germany). The assays were performed according to the manufacturer's protocol.

Evaluation of Cx43 Protein Expression

The Cx43 protein was detected in the different endometrial samples by Western blot analysis. For this purpose, plasma membranes were isolated as described previously (17). In brief, samples were homogenized for 30 seconds in homogenization buffer (20 mM of tris(hydroxymethyl)aminomethane, pH 7.5, and 250 mM of sucrose), supplemented with 10 mM of dithiothreitol, 2 mM of ethylenediaminetetraacetic acid, 5 mM of [ethylenebis-(oxyethylenenitrilo)]tetraacetic acid, pepstatin (1 mg/mL), leupeptin (1 mg/mL), and 1 mM of phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO). Large tissue particles were removed by slow centrifugation (250 × g, Type GLC-2; Sorvall) for 5 minutes. The supernatant was recovered and membranes were isolated by ultracentrifugation for 1 hour at 4°C (45,000 × g, Beckman Ultracentrifuge, Type L2-65B, Rotor Type 50 TI [Beckman, Palo Alto, CA]). The pellet was resuspended in 200 mL of homogenization buffer and the amount of Cx43 protein was determined as described previously (18).

For detection of the Cx43 protein, samples of isolated membranes were dissolved in Laemmli sample buffer (19), boiled, and loaded (30 μg of protein per lane) on 12.5% sodium dodecylsulfate-polyacrylamide gel. For better protein separation that will allow detection of the different phosphorylated forms of Cx43 (17), we recently modified the protocol, reducing the concentration of the bisacrylamide in the monomer mixture from 0.8% to 0.12%. After electrophoresis, the proteins were transferred to a nitrocellulose membrane.

The Cx43 protein was identified by specific anti-Cx43 monoclonal antibodies (Transduction Laboratories, Lexington, KY). Detection of the protein–antibody complex was performed using horseradish peroxidase–linked goat anti-

mouse antibodies followed by enhanced chemiluminesence detection reagents. Quantitation and comparison of the different samples of the autoradiograms were performed by densitometric analysis (Computing Densitometer, 420 oersted; PDI, New York, NY).

The identity of the different proteins detected by Western blot analysis as Cx43 was confirmed by depletion of the Cx43 antibodies by preincubation with a synthetic Cx43 peptide (with a sequence corresponding to amino acids 252–270; Transduction Laboratories) for 30 minutes at 37°C.
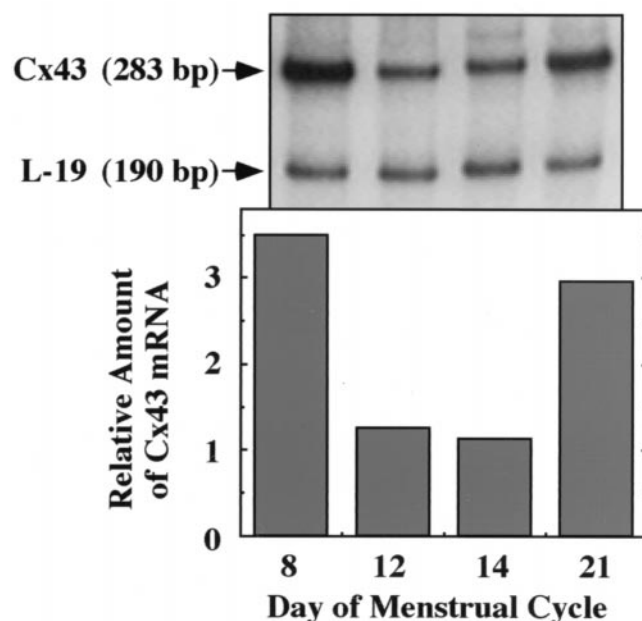
Evaluation of Cx43 Gene Expression

The Cx43 gene was detected in the different endometrial samples by reverse transcriptase polymerase chain reaction. Total RNA was extracted by the acid-guanidium-phenol-chloroform method (20) and reverse transcribed using random primers followed by polymerase chain reaction amplification. The reverse transcriptase reaction contained 50 U of Molony murine leukemia virus-reverse transcriptase, 200 μM of diethylnitrophenyl thiophosphate, 6.5 mM of $MgCl_2$, 20 U of RNAsin, 500 mg of oligo deoxythymidine, and 1.5$\times$ polymerase chain reaction buffer (Promega). The reaction was performed at 37°C for 2 hours. Fragments of the reverse transcribed Cx43 complementary DNA were amplified using a labeled nucleotide ([α-^{32}P]deoxycytidine triphosphate; Amersham) and the following pairs of primers were used: 5′-ATGGCTGCTCCTCACCAACG-3′ and 5′-GGTCGCTGGTCCACAATGGC-3′. A fragment of the ribosomal L19 complementary DNA that served as an internal control was amplified simultaneously using the following two primers: 5′-CTGAAGGTGAAGGGGAATGTG-3′ and 5′ GGATAAAGTCTTGATGATCTC-3′.

Further polymerase chain reactions were performed in the same reverse transcriptase test tube that finally contained 250 ng of each primer, 200 μM of diethylnitrophenyl thiophosphate, 2.5 mM of $MgCl_2$, 2 μCi of [α-^{32}P]deoxycytidine triphosphate, 1$\times$ polymerase chain reaction buffer (Promega), and 2.5 U of Taq polymerase. Thirty cycles and 25 cycles for L-19 and Cx43, respectively, were performed after incubation for 2 minutes at 94°C. The cycles were performed as follows: 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. This was followed by a final extension for 5 minutes at 72°C.

The radioactive products were electrophoresed on 5% nondenaturing polyacrylamide gel in 0.5$\times$ tris boric acid EDTA buffer. The gels were dried and radioactivity was determined by exposure to x-ray film. Quantitation and comparison of the different samples of the autoradiograms were performed by densitometric analysis (Computing Densitometer, 420 oersted; PDI) followed by normalization according to the internal standard.

Reverse transcriptase polymerase chain reaction analysis of the amount of Cx43 messenger RNA (mRNA) during the menstrual cycle in one study patient. bp = base pairs.

Granot. Connexin43 expression. Fertil Steril 2000.

RESULTS

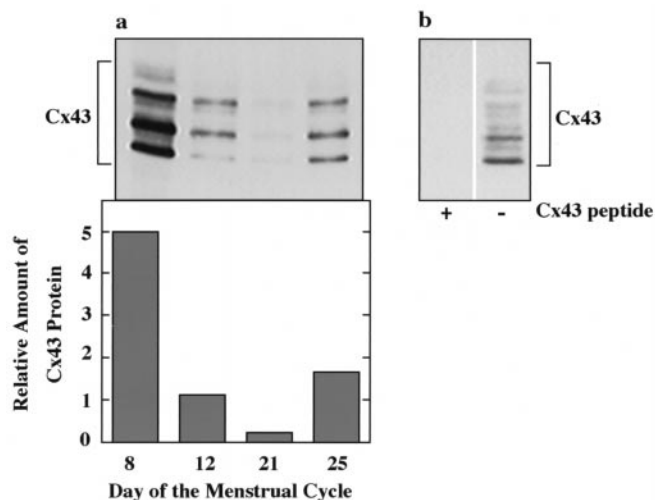### Connexin43 Gene Expression Throughout the Menstrual Cycle

The level of expression of the Cx43 gene in human endometrium observed on day 8 of the menstrual cycle decreased by 60% on day 12. A low level of the Cx43 transcript also was observed on day 14 and a high level was observed on day 21 (Fig. 1). A similar pattern of endometrial Cx43 gene expression throughout the menstrual cycle was observed in all four patients analyzed in the different experiments.

Connexin43 Protein Expression Throughout the Menstrual Cycle

Similar to Cx43 gene expression, a sharp reduction (85%) in the amount of Cx43 protein in human endometrial samples was observed between days 8 and 12 of the menstrual cycle. However, the increase in Cx43 gene expression on day 21 of the cycle, shown in Figure 1, was not accompanied by an increase in Cx43 protein expression. On the contrary, on day 21 of the cycle, the Cx43 protein was almost totally eliminated (Fig. 2A). Analysis of Cx43 protein in samples collected on day 25 of the menstrual cycle revealed that this reduction was followed by a subsequent increase in the amount of the protein (Fig. 2A).

(A): Western blot analysis of Cx43 protein expression on days 8, 12, 21, and 25 of the menstrual cycle in one study patient. (B): Depletion of the anti-Cx43 antibodies by preincubation with a Cx43 synthetic peptide completely eliminated the signal.

Granot. Connexin43 expression. Fertil Steril 2000.

The thickness of the endometrium throughout the menstrual cycle in the study patients (n = 12 for days 8, 14, and 21; n = 8 for day 12; and n = 4 for day 25). The *bars* show the SEs.

Granot. Connexin43 expression. Fertil Steril 2000.

A similar pattern of endometrial Cx43 protein expression throughout the menstrual cycle was observed in all eight patients analyzed (endometrial samples were obtained from only four patients on day 25 of the cycle). To confirm that the proteins detected by Western blot analysis actually represented the different forms of Cx43, the Cx43 antibodies were depleted by preincubation with a synthetic Cx43 peptide. This treatment completely eliminated the signal (Fig. 2B).

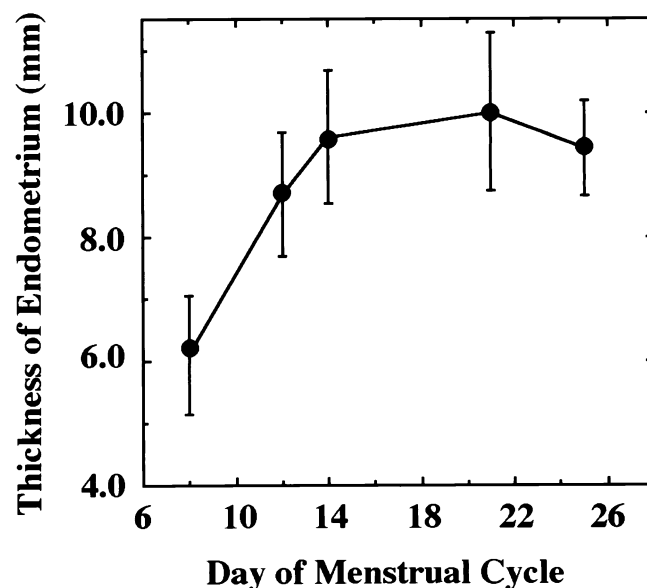### Endometrial Thickness Throughout the Menstrual Cycle

Endometrial thickness, measured in eight patients on days 8, 12, 14, 21, and 25 of the menstrual cycle, is shown in Figure 3. Endometrial thickness increased between days 8 and 12 (from 6.10 ± 0.94 mm to 8.68 ± 0.98 mm, $P=.0002$) and did not change further between days 21 and 25 of the cycle.

Hormonal Profiles

Serum gonadotropin analysis (Fig. 4) showed that the concentration of LH on day 12 (23.73 ± 3.64 IU/L) was significantly higher than that on day 8 (4.35 ± 1.08 IU/L; $P=.0045$). This elevation, which represents the midcycle surge of LH, was accompanied by a peak in the concentration of FSH (7.05 ± 1.33 IU/L and 14.84 ± 2.21 IU/L on days 8 and 12, respectively; $P=.0037$). Gonadotropin concentrations returned to basal levels on day 14 and showed no significant change on day 21.

The slight elevation in progesterone concentrations observed between days 8 and 12 of the menstrual cycle (from 2.12 ± 0.66 nmol/L to 5.21 ± 1.38 nmol/L) was followed by a sharp increase between days 14 and 21 (from 7.65 ± 2.21 nmol/L to 39.65 ± 3.91 nmol/L; $P=.0034$) (Fig. 4). High variability in serum concentrations of E_2 throughout the menstrual cycle, with no consistent pattern, was observed in all patients (range, $80-860$ pmol/L).

DISCUSSION

This study demonstrated a consistent pattern of Cx43 expression in human endometrium throughout the menstrual cycle. Using semiquantitative methods such as reverse transcriptase polymerase chain reaction and Western blot analysis, we showed that Cx43 gene and protein expression are reduced toward the middle of the cycle (days 12–14) and then increased between days 21 and 25. This pattern was observed in all 12 healthy, normally cycling women studied, 11 of whom later conceived.

Modulated expression of Cx by the stromal cells of the endometrium also has been demonstrated in rabbits and rodents. A reduction in the endometrial expression of Cx43 and Cx26 that was associated with down-regulated cell-to-cell communication was shown at preimplantation in both these species (4, 12, 13). An increase in the expression of
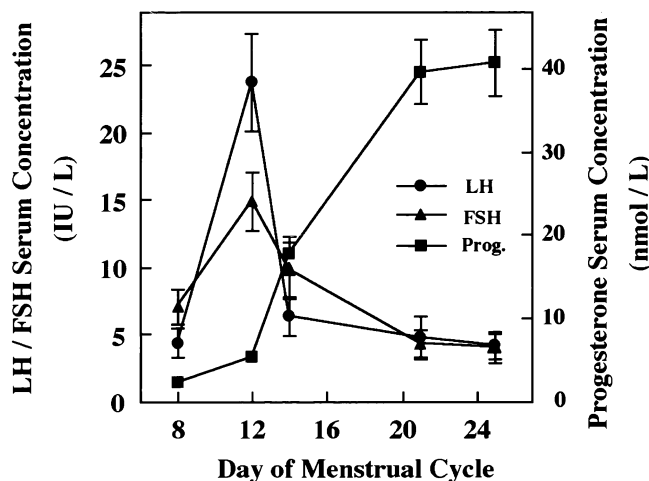
Granot. Connexin43 expression. Fertil Steril 2000.

these two Cx was shown in rat endometrium that underwent decidualization (7, 12, 21).

In agreement with these reports, we observed a sustained reduction in Cx43 protein expression between days 12 and 21 of the menstrual cycle, a period known to be part of the implantation window in humans (22). We further demonstrated an increase in Cx43 protein expression on day 25 of the menstrual cycle, which coincides with the period of decidualization (23). We suggest that, similar to rabbits and rats, the decrease in Cx43 expression in human endometrium at the time of implantation may reduce cell-to-cell communication, possibly facilitating the invasion of the trophectoderm through the stromal cells.

A pattern of Cx43 protein expression in the endometrium of patients who underwent hysterectomy was reported previously by Jahn et al. (8). This study demonstrated a very low immunohistochemical staining of Cx43 protein during the early proliferative stage of the cycle (day 8) that was increased remarkably in the middle of the cycle (days 11–15) and followed by a reduction toward the end of the cycle (days 21–26). These findings, which clearly differ from our results, apparently represent the Cx43 expression of the pathologic uteri of the patients used in that study.

Our observations, on the other hand, demonstrate the expression of Cx43 in endometrial samples from healthy, normally cycling women. Differences between the methods of tissue collection used also may explain the variations in the results obtained in the two studies. The Pipelle catheter

used in our study provides large samples of uterine tissue that were used in full for immunoblot analysis. On the other hand, the cryostat sections of endometrium used in the histochemical study (8) permit only a localized analysis that may not represent the entire tissue.

The endometrium is regulated by estrogen and progesterone. Specifically, it has been demonstrated in the rat that estrogen strongly enhances endometrial Cx43 messenger RNA expression, whereas progesterone suppresses it (7). The increase in the amount of the Cx43 transcript that we observed on day 21 and that occurs in conjunction with an increased concentration of serum progesterone seems to contradict the findings of Jahn et al. (8). However, it strongly confirms our previous finding that LH downregulates the expression of Cx43 gene and protein in rat ovarian follicles (17, 24). We therefore suggest that the midcycle decrease in human endometrial Cx43 expression demonstrated in our study may reflect the inhibitory effect of LH on this Cx. This assumption is supported by previous findings that endometrial stromal cells express receptors for LH and hCG (25).

The modulations in Cx43 gene expression that occurred toward the middle of the menstrual cycle were associated with parallel changes in the protein, suggesting that this Cx is regulated at the transcriptional level. The increase in Cx43 gene expression on day 21 that was not associated with an increase in Cx43 protein expression suggests a conversion from transcriptional to translational regulation of Cx43 during the last third of the menstrual cycle. Indeed, further analyses of endometrial samples taken at a later stage in the menstrual cycle revealed delayed protein synthesis. The mechanism of regulation of Cx43 protein synthesis in human endometrium toward the end of the menstrual cycle is presently unknown.

In conclusion, we demonstrated a consistent pattern of Cx43 expression in human endometrium throughout the menstrual cycle. This pattern may serve as a biochemical reference for normal cyclicity of the endometrium, and it may be useful in predicting the chances of successful implantation. This information may be of major clinical importance in the diagnosis of infertility.

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