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Local injury of the endometrium induces an inflammatory response that promotes successful implantation

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Abstract

Objective—To study whether an injury-induced inflammation might be the mechanism underlying the favorable effect of endometrial biopsy on the implantation rate in in vitro fertilization (IVF) patients.

Design—Controlled clinical study.

Setting—A medical center IVF unit and a research institute.

Patient(s)—Women undergoing IVF who had previous failed treatment cycles.

Intervention(s)—Endometrial samples were collected from two groups of patients on day 21 of their spontaneous menstrual cycle. The experimental, but not the control group underwent prior biopsy treatment on days 8 or/and 11 to 13 of that same cycle.

Main Outcome Measure(s)—Abundance of immune cells, cytokines/chemokines level, correlation between these parameters and pregnancy outcome.

Result(s)—A statistically significantly higher amount of macrophages/dendritic cells (HLA-DR⁺ CD11c⁺ cells) and elevated proinflammatory cytokines, tumor necrosis factor- α (TNF- α), growth-regulated oncogene- α (GRO- α), interleukin-15 (IL-15), and macrophage inflammatory protein 1B (MIP-1B), were detected in day-21 endometrial samples of the experimental group. A direct stimulatory effect of TNF- α on MIP-1B, GRO- α , and IL-15 messenger RNA (mRNA) expression was demonstrated. A positive correlation was found between the levels of macrophages/dendritic cells, MIP-1B expression, and TNF- α expression and the pregnancy outcome.

Conclusion(s)—A biopsy-induced inflammatory response may facilitate the preparation of the endometrium for implantation. Increased MIP-1B expression could possibly serve for prediction of implantation competence.

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Keywords

Cytokines; endometrium; immune cells; inflammation; local injury

Implantation of the embryo, which is a prerequisite for successful pregnancy, can only take place in a receptive uterus. In humans, the uterus becomes receptive during the midsecretory phase of the menstrual cycle (days 19 to 23), commonly known as the window of implantation (WOI). It is assumed that inadequate uterine receptivity is responsible for approximately two-thirds of implantation failures (1). Although many fertility disorders have been overcome by a variety of assisted reproductive techniques, implantation remains the rate-limiting step for the success of in vitro fertilization (IVF).

We have previously demonstrated that endometrial biopsies taken during the spontaneous cycle preceding IVF treatment more than double the rates of implantation, clinical pregnancy, and live birth (2). The favorable influence of local injury of the endometrium was later confirmed by others (3–5). The association of mechanical manipulation with decidual formation has been reported previously in rodents (6,7). These early observations in rodents, combined with our findings and those of others in humans, suggest that local injury of the endometrium facilitates successful implantation.

Thus, we hypothesized that the success of implantation in biopsy-treated IVF patients is secondary to the development of an injury-induced inflammatory reaction. A high level of endometrial proinflammatory cytokines, such as interleukin-6 (IL-6), leukemia inhibitory factor (LIF), and tumor necrosis factor- α (TNF- α), characterize early implantation (8). These cytokines can be secreted by the endometrial cells as well as by cells of the immune system that are recruited to the site of implantation. In humans and mice, large populations of decidual leukocytes infiltrate the implantation site. Of these cells, 65% to 70% are uterine-specific natural killer cells (8), which have been shown to be essential for the establishment of an adequate decidua (9,10). An additional 10% to 20% of the decidual leukocytes consist of macrophages and dendritic cells (DCs) (11,12), which remain in the decidua throughout pregnancy and secrete growth factors, chemokines, and cytokines regulating blastocyst implantation, angiogenesis, placental development, and decidual homeostasis (13,14). A recent study conducted in our laboratory demonstrated that depletion of uterine DCs leads to impaired decidualization and to embryo resorption in mice (15). This result is supported by a previous report of a reduction in spontaneous embryo resorption after inoculation with DCs (16).

We examined the hypothesis that local injury of the endometrium provokes an inflammatory reaction that, in turn, increases the amount of DCs and macrophages that may play a role in the development of a receptive endometrium. Furthermore, we elucidated the correlation between injury-driven inflammation and pregnancy outcome.

MATERIALS AND METHODS

Patients and Sample Collection

Sixty-four 22- to 39-year-old IVF patients who had menstrual cycles of 28 to 30 days and a good response to hormonal stimulation were selected. Patients with endometriosis and hydrosalpinx were excluded. Patients were divided into two groups, experimental ($n = 42$) and control ($n = 22$). The age of the women and the number of previous failed IVF cycles (32.4 ± 3.4 vs. 33 ± 3.9 , $P = .43$ and 3.7 ± 3.1 vs. 4.2 ± 1.8 , $P = .08$, respectively) in the groups was similar.

Collection of endometrial samples from patients of both groups was performed during the WOI, days 20 to 23 of their spontaneous menstrual cycle. A sample of the functional layer of the endometrium was retrieved using a biopsy catheter (Pipelle de Cornier, Prodimed, Neuilly-en-Thelle, France), as previously described elsewhere (2). Patients in the experimental group underwent prior biopsies on days 8 to 9 and/or 11 to 13 of the same cycle. Each biopsy sample was divided into two parts; one was placed in cold phosphate-buffered saline (PBS) for flow cytometry analysis, and the other was immediately plunged into liquid nitrogen and later was used for RNA extraction, multiple-cytokine analysis, and histology analysis to confirm the menstrual cycle phase. For cell culture experiments, fresh endometrial fragments were collected in culture medium.

The protocol of this study was approved by the Kaplan Medical Center Review Board on the use of human subjects in medical research in accordance with the Helsinki Declaration and by the Israeli Ministry of Health in Jerusalem. All patients underwent IVF/intracytoplasmic sperm injection (ICSI) treatment in the following cycle.

Single Cell Isolation and Flow Cytometry Analysis (FACS)

Endometrial samples were minced into fragments of 1 mm³ and digested for 30 minutes at 37°C with 1 mg/mL of collagenase type IV (Sigma-Aldrich, St. Louis, MO), 0.2 mg/mL of DNase (Roche Applied Science, Mannheim, Germany), and 1 mg/mL of bovine serum albumin in PBS with MgCl and CaCl (Sigma-Aldrich). The cell suspension was passed through a mesh, washed in PBS, treated with ACK buffer (0.01 M KHCO₃-buffered 0.16 M, NH₄Cl, 0.1 mM ethylenediaminetetraacetic acid [EDTA]) to remove red blood cells, washed and resuspended in PBS. Isolated cells were incubated with human immunoglobulin for 10 minutes and stained with fluorescent antibodies against specific antigens of different immune cell populations: anti-CD14 fluorescein isothiocyanate (FITC) (Miltenyi Biotech, Bergisch Gladbach, Germany), a specific marker for macrophages; anti-CD11c PE (BD Biosciences, San Jose, CA) for myeloid type-DCs; anti-CD45 FITC for leukocytes; anti-HLA-DR allophycocyanin (APC), a common marker for macrophages and DCs; and anti-CD56 PerCP/Cy5.5 (Biolegend, San Diego, CA) for natural killer cells. After 30 minutes of incubation at 4°C, the cells were washed, resuspended in fluorescence-activated cell sorter (FACS) buffer, and analyzed on FACSsort flow cytometer (Becton-Dickinson, Franklin Lakes, NJ) using CellQuest software (Becton-Dickinson). Unlabeled cells and the respective isotype antibodies were used as a control.

Cytokine Analysis of the Endometrial Samples

Total RNA was extracted using an RNA isolation kit (Zymo Research, Orange, CA), and first-strand complementary DNA (cDNA) was synthesized from 2 µg of RNA using reverse transcriptase (Promega, Madison, WI). Expression of the selected genes was determined by quantitative real-time polymerase chain reaction (qRT-PCR) using DNA Master Plus SYBR green I (Finnzymes Oy, Espoo, Finland) on the iCycler (Bio-Rad Laboratories, Hercules, CA) and was normalized to hypoxanthine phosphoribosyltransferase 1 (HPRT1). Protein concentrations of the cytokines were analyzed using Luminex 100 IS (Upstate Biotechnology, Charlottesville, VA) according to the manufacturer's instructions.

In Vitro Regulation of Cytokine Expression by TNF-α

Fresh endometrial fragments, collected in sterile medium, were rinsed to remove blood cells then were minced and incubated in Dulbecco's modified Eagle's medium (DMEM)/F12 containing 10% fetal bovine serum (FBS), 2.5 mg/mL of collagenase type IV, and DNase for 1.5 hours at 37°C. The digested cells were filtered through a 40-µm cell strainer (BD Biosciences). Endometrial stromal cells (ESCs) that passed through were collected. Either ESCs or epithelial cell line (ECC-1) were cultured in a density of 3×10^5 per well in six-

well plates for 24 hours in phenol red-free DMEM/F12 medium with 10% charcoal-stripped FBS. After an overnight starvation in serum-free medium, the cells were treated with different concentrations of TNF- α for 24 hours. The RNA was extracted using the PerfectPure RNA Cell Kit (5Prime, Inc., Gaithersburg, MD) and analyzed by qRT-PCR.

Statistical Analysis

Comparison between samples from days 8 to 12 and day 21 within the experimental group was performed using the Wilcoxon signed rank test. Correlation between mRNA levels of the different cytokines was analyzed by Spearman correlation coefficient R . A nonparametric Mann-Whitney U test was employed for comparison between day-21 samples of the control and the experimental groups and between pregnant patients and those who did not conceive despite high-quality embryos transfer (GraphPad Software, Inc., San Diego, CA). Further multiple logistic regression analysis for identifying the best predictors for embryo implantation and pregnancy was performed using SAS software (SAS Institute, Inc., Cary, NC). In vitro experiments were statistically analyzed using an unpaired Student's t -test. $P \leq .05$ was considered statistically significant.

RESULTS

Analysis of the cytokine/chemokine concentrations in the endometrium showed an increase in the protein levels of growth-regulated oncogene- α (GRO- α), IL-15, and macrophage inflammatory protein 1B (MIP-1B) in day-21 samples of the experimental group as compared with that of the control group ($P=.02$, $P=.006$, $P=.031$, respectively; Fig. 1A–C). The up-regulation of MIP-1B was confirmed at the mRNA level (see Fig. 1D).

The expression of TNF- α , an early proinflammatory cytokine associated with tissue injury and repair, was also significantly higher in day-21 samples of the biopsy-treated group as compared with that of the control group (see Fig. 1E). Furthermore, the expression of TNF- α positively correlated with MIP-1B expression levels ($R=0.67$, $P<.0001$). To better characterize this correlation, the effect of TNF- α on endometrial cells was tested in freshly isolated primary cultured human ESCs and ECC-1 cells. We found that TNF- α increased MIP-1B expression in a dose-dependent manner in both stromal and epithelial cells, whereas the expression of mucin 1 (MUC1), an endometrial surface protein, was up-regulated as expected only in the epithelial cells (see Fig. 1F, G). Moreover, TNF- α induced an increase in the expression of the two other biopsy-induced proinflammatory cytokines, GRO- α and IL-15, in stromal and epithelial cell, respectively (see Fig. 1F, G).

To identify and evaluate the abundance of macrophages/DCs, we applied FACS analysis. The leukocyte region was defined using characteristic size and granularity parameters (R1; Fig. 2A) as well as by positive CD45 staining (R2; see Fig. 2B). In this region, all HLA-DR⁺CD56[−] cells (R3) showed positive staining for CD11c (see Fig. 2C, D) and exhibited heterogeneity in size and granularity (data not shown), which characterizes DCs and macrophages (12). In addition, staining with anti-CD14 revealed that $85.2 \pm 6.8\%$ of these HLA-DR⁺CD11c⁺ cells were macrophages ($n = 37$; see Fig. 2E, F). The abundance of macrophages/DCs was calculated as the percentage of HLA-DR⁺CD11c⁺ cells out of the total endometrial leukocytes (CD45⁺ cells). This analysis revealed a statistically significantly higher amount of macrophages/DCs in the day-21 samples of the biopsy-treated patients as compared with days 8 to 12 in this same group ($29.4 \pm 14.5\%$ and $17.6 \pm 6\%$, respectively) as well as with day-21 samples from the control group ($19.2 \pm 5.6\%$; see Fig. 2G, H).

We also tested the effect of biopsy treatment on the expression of osteopontin (OPN) and vascular endothelial growth factor (VEGF). These proteins are produced by both

macrophages/DCs and the receptive endometrium, and they have a role in implantation and angiogenesis (17,18). Although VEGF expression remained unchanged, OPN mRNA and protein levels were significantly higher in day-21 samples from the biopsy-treated group as compared with the samples from the controls (Fig. 3). We also observed a correlation between OPN and MIP-1B expression ($R=0.56$; $P<.0001$).

To further study the effect of biopsy-induced inflammation on uterine receptivity, the patients were divided into two subgroups according to the pregnancy outcome after IVF treatment in the subsequent cycle. We found that achievement of pregnancy was associated with a higher abundance of HLA-DR⁺CD11c⁺ cells as well as with a higher expression of MIP-1B, TNF- α , and OPN ($P<.05$; Fig. 4). It is important to mention that no significant differences in age (31.6 ± 3.5 vs. 32.4 ± 4.08 , $P=.5$) or number of previous failing cycles (4.8 ± 3.7 vs. 3.2 ± 2.4 , $P=.13$) were recorded between pregnant and nonpregnant patients. The percentage of ICSI as the mode of insemination was somewhat, but not significantly, higher in the group of pregnant as compared with nonpregnant patients (88% vs. 77%, respectively; Chi-Square test), indicating that male factor infertility did not affect the IVF outcome. We used logistic regression to detect the best predictors for pregnancy. This analysis revealed that MIP-1B has the highest potential to serve as a biomarker for prediction of implantation competence after IVF treatment—the higher its level, the higher the probability of becoming pregnant ($P=.038$).

DISCUSSION

Our results demonstrate for the first time that endometrial biopsy triggers an inflammatory response characterized by an influx of macrophages/DCs (HLA-DR⁺CD11c⁺ cells) as well as by an increase in proinflammatory cytokines. We also show that the abundance of these cells and the expression of cytokines positively correlate with pregnancy outcome. Taken together, these findings and the previous observation of the beneficial effect of biopsy treatment on implantation (2–5) imply that the injury-induced inflammatory response facilitates the transition of a nonreceptive uterus into a receptive uterus, a reaction that does not take place in patients who did not receive such endometrial treatment.

It is interesting that the favorable effect on endometrial receptivity was manifested in the cycle that followed biopsy treatment. This long-term effect may rely on the fact that monocytes recruited to injured sites are long-lived and reside in some tissues for months. During this time they can differentiate into tissue-resident macrophages/DCs in response to cytokines that are expressed during the WOI such as TNF- α (19–21). In this context, it is also important to note that the reduction in endometrial thickness during menstruation is primarily due to the loss of fluid and shrinkage of the spongy layer, leaving most of the stroma and apparently the embedded immune cells intact. Regeneration of the endometrium occurs from a residuum of the functional rather than from the basal layer (22).

The analysis of cytokines in the endometrial samples recovered from the biopsy-treated patients revealed an increased expression of Gro- α , IL-15, MIP-1B, and TNF- α with a strong correlation between TNF- α and MIP-1B. Our in vitro experiments further showed a TNF- α -induced increase in the expression of MIP-1B. These results imply that in biopsy-treated patients the expression of MIP-1B may be mediated by TNF- α . These two cytokines were previously detected during the WOI (23), suggesting the role of inflammation in development of a receptive endometrium. Our findings strongly support this idea, showing a positive correlation between the increased expression of TNF- α and MIP-1B after endometrial biopsy treatment and the clinical pregnancy outcome.

Macrophages and DCs which are present in human endometrium were shown to play a role in decidualization and implantation. (12,13,15,24,25). Furthermore, we have shown that macrophages function as support cells by facilitating trophoblast invasion in mice (25). Indeed, our results demonstrated an increased abundance of macrophages/DCs in the endometrium of patients after biopsy treatments. Because MIP-1B is responsible for the attraction of these immune cells (26,27) we propose that the increased receptivity of the uterus after biopsy treatments might be mediated by immune cells recruited by MIP-1B. Moreover, the presence of these cells in the endometrium positively correlated with the clinical pregnancies obtained in the subsequent IVF treatment. These results strongly support the assumption that macrophages/DCs play an important role in the preparation of a receptive endometrium. Macrophages and DCs have the ability to secrete an array of cytokines/chemokines and enzymes that are involved in tissue remodeling and angiogenesis (28,29). In addition, these molecules may possibly act as mediators of the immune cells that potentially target the luminal epithelium (8), thus contributing to the acquisition of endometrial receptivity.

Osteopontin, an adhesive molecule, has been proposed as a bio-marker for a receptive endometrium because it is up-regulated in human endometrium during the WOI (30). The up-regulation of OPN in biopsy-treated patients as well as the positive correlation between its expression and pregnancy outcome presented herein further supports the favorable effect of biopsy treatment on uterine receptivity. Osteopontin is also known as a proinflammatory cytokine secreted by the endometrium and by immune cells (18,31). This protein recruits and activates macrophages and DCs (32,33), an effect that was recently shown to be mediated by MIP-1B (34). Therefore, on top of its function as an adhesive molecule, OPN may positively regulate the secretion of MIP-1B, thus contributing to the recruitment of macrophages/DCs.

Most exciting is the identification of MIP-1B as a potential bio-marker for predicting implantation competence in patients with repeated implantation failures. Evaluating uterine receptivity by using biomarkers such as MIP-1B will improve IVF treatment. The high probability of implantation would facilitate the transfer of a single embryo, avoiding the subsequent, often severe complications of multiple pregnancies. Alternatively, prediction of a low chance for successful implantation could enable a recommendation that IVF may not be the immediate solution for these patients. A prospective clinical trial with a larger group of patients is needed to gain statistical power for the establishment of MIP-1B as a predictor of implantation with high probability.

Our results are compatible with the following series of events. Local injury by endometrial biopsy promotes an inflammatory response. Proinflammatory cytokines such as $\text{TNF-}\alpha$, produced by the wounded endometrium, stimulate the secretion of other chemokines/ cytokines which, in turn, recruit macrophages/DCs to the site of implantation. These immune cells enhance the inflammatory reaction and may trigger the uterine epithelium to produce molecules that interact with the blastocyst, facilitating its apposition and attachment to the uterine wall. Based on the strong correlation of pregnancy outcome with the increase in the abundance of macrophages/DCs and cytokine expression, we suggest that biopsy-induced inflammation may facilitate the preparation of a receptive endometrium in IVF patients with repeated implantation failure. Moreover, we propose that MIP-1B could possibly be used to predict implantation competence and/or the success of the biopsy-treatment in IVF cycles.

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FIGURE 1.

Evaluation of the cytokine/chemokine profile in the endometrial samples. Protein levels of (A) growth-regulated oncogene- α (GRO- α), (B) interleukin-15 (IL-15), (C) macrophage inflammatory protein 1B (MIP-1B) were determined by multiple cytokine Luminex analysis. (D, E) Relative MIP-1B and tumor necrosis factor- α (TNF- α) messenger RNA (mRNA) levels were tested by quantitative real-time polymerase chain reaction. The box plot horizontal lines represent the median and the 25th to 75th percentile. Comparison between days 8 to 12 and 21 within the experimental group was performed by Wilcoxon's signed rank test. Comparison between day-21 samples of the experimental and control groups was performed using Mann-Whitney test (** $P < .01$, * $P < .05$). The effect of TNF- α on the expression of MIP-1B, MUC1, Gro- α , and IL-15 was tested in (F) freshly isolated endometrial stromal cells (ESC) from day-21 control samples and in (G) epithelial cell line ECC-1. Results of in vitro experiments are mean $\pm$ standard error of the mean from three independent experiments. Endometrial stromal cells were prepared from three different tissues. *, #, and † are significantly different from their respective control ($P < .05$; student t-test).

FIGURE 2.

Characterization of macrophages/dendritic cells (DCs) in the endometrial samples by flow cytometry. Leukocytes region was defined using (A) characteristic size (FSC) and granularity (SSC) parameters (R1) as well as by positive staining with (B) anti-CD45 (R2). (C) The HLA-DR⁺CD56⁻ cells (R3) within leukocyte region (R1 and R2) that exhibited a positive staining for (D) CD11c were defined as macrophages/DCs. (E) HLA-DR⁺CD11c⁺ cells (R4) were separated to (F) macrophages and DCs according to CD14 staining. (G) A representative analysis of HLA-DR⁺CD11c⁺ cells in endometrial samples from days 8 to 12 and day 21 of the experimental group and the day-21 sample of the control group. (H) The abundance of HLA-DR⁺CD11c⁺ cells was calculated of total leukocytes (R1 and R2). The box plot horizontal lines represent the median and the 25th to 75th percentile. Comparison between days 8 to 12 and 21 within the experimental group was performed by Wilcoxon's test signed rank test. Comparison between the day-21 samples of the experimental and control groups was performed using Mann-Whitney test. *** $P < .001$.

FIGURE 3.

Expression of osteopontin (OPN) and vascular endothelial growth factor (VEGF) in the endometrial samples. The box plot horizontal lines represent the median and the 25th to 75th percentile of (A) VEGF messenger RNA (mRNA) levels, (B) OPN mRNA, and (C) protein levels. * $P < .05$, ** $P < .01$ (Mann-Whitney test).

FIGURE 4.

Comparison of the abundance of (A) macrophages/dendritic cells (HLA-DR⁺CD11c⁺ cells), (B) messenger RNA (mRNA) levels of macrophage inflammatory protein 1B (MIP-1B), (C) tumor necrosis factor- α (TNF- α), and (D) osteopontin (OPN) between pregnant (+) and not pregnant (-) IVF patients. The box plot horizontal lines represent the median and the 25th to 75th percentile. * $P < .05$ (Mann-Whitney test).