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Correlation of NK Cell Activation and Inhibition Markers with NK Cytoxicity Among Women Experiencing Immunologic Implantation Failure After In Vitro Fertilization and Embryo Transfer

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***Purpose*:** The pivotal event in determining successful from unsuccessful cycles after in vitro fertilization is implantation. The purpose of this study was to compare the percentage of circulating NK cells expressing activation and inhibition markers between infertile and fertile control women and to determine the correlation between these markers and those of the NK cytotoxicity activation assay. Lastly, we wish to determine the ability of each of these markers to predict pregnancy outcome after IVF/ET (in vitro fertilization/embryo transfer).
***Methods*:** Blood samples from 22 infertile women undergoing IVF/ET during the November 2001 cycle were drawn on cycle Day 9 and analyzed for expression of CD69+, HLA-DR, CD161+, CD94+, and CD158a+ as well as NK cytotoxicity using immunoflluorescent labeling and flow cytometry. Results were compared with those from 26 fertile control women and correlated to pregnancy outcome that of cycle.
***Results*:** Infertile women had significantly higher expression of NK cell activation markers of CD69+ and CD161+ than fertile women. NK cytotoxicity correlated inversely with expression of NK cells bearing the inhibition marker of CD94+. None of the successfully pregnant women of that cycle had elevated levels of NK cytotoxicity whereas 50% of those experiencing a chemical pregnancy loss and those not becoming pregnant had elevated levels of NK cytotoxicity.
***Conclusions*:** Immunologic markers can identify mechanisms involved in implantation failure. Activation markers of CD69+ and CD161+ expressed on NK cells as well as NK cytotoxicity can be added to the previously reported risk factors for immunologic implantation failure.

KEY WORDS: Implantation failure; NK cell activation and inhibition markers; NK cytotoxicity.

INTRODUCTION

Lack of successful implantation accounts for 75% of conceptions that are lost (1) and represents the most frequent cause of in vitro fertilization (IVF) and embryo transfer (ET) failures. Unsuccessful implantation can be the consequence of abnormalities within the conceptus or the uterus (2). Abnormalities within the conceptus are largely reflected as chromosomal aberrations. Data from preimplantation genetic studies show that while the frequency of chromosomal abnormalities increases with advancing maternal age (3), the overall percentage of karyotypic abnormalities among preimplantation embryos is around 60% (4). If the probability of karyotypically abnormal conceptus is 60% and three fertilized oocytes are

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transferred, the probability of at least one normal fertilized egg is 78%, a value much greater than the most recent estimate of the likelihood of live birth after embryo transfer of 27.9% (5). The decrease in expected implantation rate from karyotypically normal embryos could be the result of abnormalities within the uterus. While anatomic and hormonal deficiencies have been cited as uterine causes of implantation failure, data are accumulating supporting a role of the immune system in successful (6,7) and unsuccessful (8–10) pregnancy. Lymphocytes are present in human decidua around the time of implantation (11). The majority of the lymphocyte population consists of NK cells with the specific phenotype CD56+/CD16− (12). Peripheral blood NK cells (13–17) and NK cytotoxicity (17) are increased in women experiencing implantation failure. Several receptors on NK surfaces contribute to NK function. These receptors can be either inhibitory or activating and the balance between them controls NK cytotoxicity. Activation of cytotoxicity is detrimental to embryo implantation and inhibition of cytotoxicity is associated with successful implantation (18). The inhibitory receptors on NK surfaces are either of the KIR family (such as CD158a) or of the C-type lectin family (such as CD94). CD69 and CD161 and HLA-DR are among the activating receptors.

The purpose of the current study is to compare the percentage of circulating NK cells expressing CD69, CD161, HLA-DR, CD94, and CD158a between infertile and control women and to determine the correlation between these results and those of the NK cytotoxicity activation assay. Lastly, we wish to determine the ability of each of these markers to predict pregnancy outcome after IVF/ET.

MATERIALS AND METHODS

Blood Specimens

Blood from 22 women undergoing IVF/ET at the Sher Institute for Reproductive Medicine in Chicago during the November 2001 cycle was drawn prospectively on cycle Day 9 of gonadotropin stimulation and analyzed for the percentage of circulating NK cells expressing CD69, CD161, CD94, and CD158a and NK cytotoxicity. The results of these assays were compared with results obtained from 26 fertile control women and correlated with pregnancy outcome of that cycle.

NK Cytotoxicity Assay

The determination of natural killer cell function was performed by flow cytometry using a previously described technique (19). Briefly, K562 cells (a human erythroleukemia cell line) were grown as stationary cultures at 37°C in 5% CO_2. The cells were subcultured for 3 days before the assay, to be certain they were in the log phase. Before use in the assay, cells were adjusted to 1×10^6 and labeled with 10 μL/mL of 3 mmol/L 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) per milliliter for 20 min at 37°C, 5% CO_2. Effector cells were isolated from the buffy coat of heparinized blood using the Ficol–Hypaque centrifugation. Target cells at the standard concentration and effector cells at various dilutions (1:1, 1:2, 1:4) were added to create effector/target ratios from 50:1 down to 12:1. The mixture was centrifuge for 30 s at 1000*g* to pellet target and effector cells. After 3 h of incubation at 37°C, 5% CO_2 100 μL of propidium iodide (PI) (0.01% working solution in culture medium) was added to the tubes to stain the dead cells. Data were collected for analysis on the Becton–Dickinson FACSCaliber flow cytometer, using the Cell Quest software. The spontaneous lysis was subtracted from the actual lysis for each sample. Based upon the control population, increased NK killing activity was defined as greater than 10% killing.

NK Activation and Inhibition Marker Assays

Peripheral blood was collected in heparinized (green topped) tubes and 100 μL is incubated for 15 min with anti-CD56 FITC, anti-CD16 APC and anti-CD69, anti-CD161, anti-CD94, and anti-CD158a PE monoclonal antibodies (mAb). Control mAbs include mouse IgG1 FITC, IgGPE, and IgG APC. After lysing the erythrocytes and wash, the samples were run with BD FASCaliber flow cytometer. Results were analyzed using a Cell Quest three or four color protocol.

Statistical Analysis

Results of the NK activation and inhibition markers were compared between infertile women undergoing treatment with IVF/ET and fertile control women using Fisher's Exact test. Comparisons of results from the NK cytotoxicity assay and the NK activation and inhibition markers were made using regression analysis with Pearson correlations. Comparisons of

results from the NK cytotoxicity assay, NK with pregnancy outcome that cycle were done using Mann–Whitney test. Differences were considered significant at $P < 0.05$.

RESULTS

Patients

The mean age of the patients was 36.7 years. The majority of the patients had a diagnosis of unexplained infertility (18/22 = 81%) and other indications for IVF/ET were tubal factor (3/22 = 14%) and endometriosis (1/22 = 5%). Nine women (40%) had previous IVF cycles that had failed.

NK Activation and Inhibition Markers

Values for NK activation and inhibition markers were Gaussian in distribution and the normal range was determined by the evaluating the mean ±2 standard deviations of the mean for the control population. Normal ranges for NK activation and inhibition markers are shown in Table I.

Figure 1 compares the percentage of abnormal results of expression of activation and inhibition markers among 22 infertile women undergoing in vitro fertilization with 26 fertile control women. Infertile women had significantly elevated circulating NK cell expressing surface activation markers CD69 ($P < 0.007$) and CD161 ($P < 0.02$) compared with fertile women. Significant elevations of CD69 were noted in CD56+16− cells while those of CD161 were CD56+16+ cells. No significant elevations of CD56+16+HLA-DR+ or CD56+16−HLA−DR+ cells were observed. When expression of NK cell surface inhibition markers of CD94 and 158a were compared between infertile and fertile women, no differences were seen (data not shown).

Table I. Normal Range for NK Cell Activation and Inhibition Markers Based on Mean Value ± 2 Standard Deviation from the Mean of Fertile Control Women

Marker	Normal range (% expression)
Activation	
CD56+16+69+	6–35
CD56+16−69+	2–34
CD56+16+HLA-DR+	7–73
CD56+16−HLA-DR+	5–89
CD56+16+161+	16–90
CD56+16−161+	4–74
Inhibition	
CD56+16+94+	21–94
CD56+16−94+	4–90
CD56+16+158a+	7–65
CD56+16−158a+	1–16

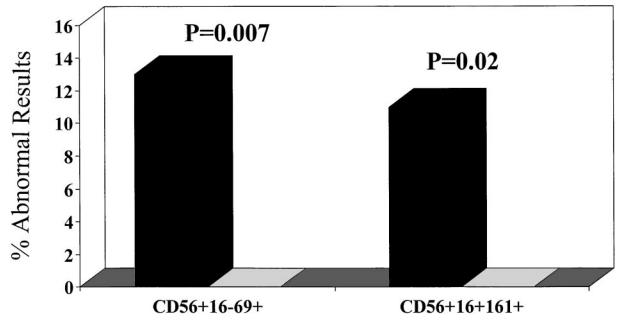

Fig. 1. Percentage of elevated expression of activation markers of NK cells among infertile women undergoing treatment with IVF/ET (black bars) compared with fertile women (gray bars).

NK Cytotoxicity Assay

When percentage of elevated expression of NK surface activation and suppression markers were compared with results of the NK cytotoxicity assay, the only correlation observed was a negative correlation with CD94 (Fig. 2).

Pregnancy Outcome

The pregnancy rate for the 22 women undergoing IVF/ET of that cycle was 55% (12/22). Half (6/12) of those who became pregnant or 27% (6/22) of the women who cycled experienced a chemical pregnancy loss (two rising serum beta hCG concentrations

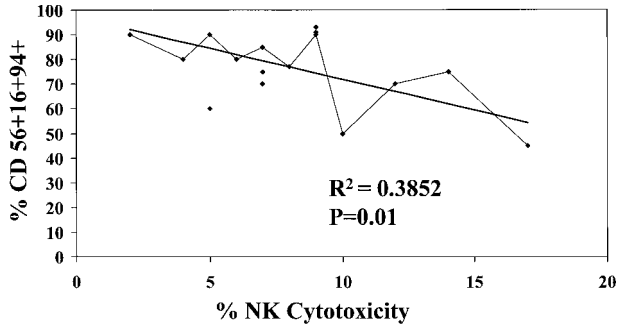

Fig. 2. Regression analysis using Pearson's correlation to compare peripheral expression of the NK inhibition marker of CD94+ and NK cytotoxicity.

Table II. Comparison of Pregnancy Outcome with Percentage of Expression of Abnormal NK Cytotoxicity Among Infertile Women Undergoing Treatment with IVF/ET

	Pregnant ($n = 6$)	Chemical pregnancy ($n = 6$)	Nonpregnant ($n = 10$)
Percentage of NK cytotoxicity >10%	0%*	50%	50%

* $P < 0.05$.

10–14 days after embryo transfer and no embryonic sac visible on ultrasongraphic examination at 5 weeks gestation). The remainder of the women continued their pregnancy until term or well into their third trimester. When the expression of NK surface activation and suppression markers were compared with pregnancy outcomes, no differences were noted between successfully pregnant and unsuccessfully pregnant women.

None of the successfully pregnant women had abnormal results in the NK cytotoxicity assay (>10%), whereas 50% of the women experiencing chemical pregnancy loss and 50% of women not achieving pregnancy had abnormally high percentage (>10%) of NK cells with cytotoxic activity (Table II).

DISCUSSION

The results of the current study show that infertile women undergoing treatment with IVF/ET have a higher percentage of elevated expression of circulating NK cell activation markers and cytotoxicity activity. Elevated circulating levels of NK and activated NK cells among infertile women compared with control women have been previously published (16–20). Women with a history of multiple prior implantation failures after IVF/ET characterized by a negative pregnancy test (13,15) and chemical pregnancies (16) after embryo transfer demonstrated significantly higher circulating levels of CD56+ NK cells than normal fertile controls. Moreover, when circulating levels of CD56+ NK cells were measured on the day of embryo transfer, the percentage of CD56+16− and CD56+16+ were significantly higher among women who failed to implant that cycle compared with those who implanted (17). All of these studies support the concept that NK cells have an important role in the success or failure of implantation. In addition, elevated percentages of circulating NK cells during pregnancy have been shown to predict loss of karyotypically normal pregnancies (10). The increased prevalence of circulating NK cells among women experiencing implantation failure has been suggested to indicate an association with loss of karyotypically normal preimplantation embryos (16).

NK cells secrete cytokines (18). Some cytokines enhance pregnancy growth and development and some are embryotoxic (18,21,22). Cytokines shown to be embryotoxic have included the Th 1 cytokines that are secreted by NK cells when they are activated (22). Activation markers of NK cells include CD69, HLA-DR, and CD161. A previous report demonstrated a significant increase in CD69+ expression on CD56+ NK cells in women with infertility as compared with controls (20). In addition to elevated percentages of circulating NK cells expressing CD69+, the current study showed a significant increase in the cell surface activation marker of CD161+ among women undergoing IVF/ET for treatment of infertility. A difference in the distribution of the activation markers was observed. CD69+ expression was significantly increased on CD56+16− cells while the significant elevation of expression of CD161+ was limited to CD56+16+ cells (Fig. 1). The ligands for CD69+ and CD161+ are unknown, but it can be speculated that an excess of activated NK cells might play a detrimental role in successful implantation. No differences in expression of NK cell surface inhibition markers of CD94 and CD158a were observed when women experiencing implantation failure were compared with fertile women. In contrast, when women with a history of recurrent miscarriages were studied, a significant decrease in CD94+ expression was noted (20). This observation suggests NK inhibitory activity may play a role in successful pregnancy a little later than the beginning of the implantation period.

The NK cytotoxicity assay predicted pregnancy outcome after IVF/ET. None of the successfully pregnant women had abnormal results in the NK cytotoxicity assay (>10%), whereas 50% of the women experiencing chemical pregnancy loss and 50% of women not achieving pregnancy had abnormally high percentage (>10%) of NK cells with cytotoxic activity (Table II). These results are in agreement with a previous report that demonstrated increased NK cytotoxicity measured on the day of embryo transfer was associated with decreased implantation rate of that cycle (17). NK cytotoxicity correlated inversely with expression of CD94+ (Fig. 2) suggesting that the cytotoxic activity might be the result of lack of inhibitory mechanisms. Yet expression of CD94+ was not significantly decreased in women who achieved successful pregnancy compared with those who did not in that

cycle. Two explanations for these discrepant results can be offered: 1) the sample size was too small to show a significant difference between pregnant and nonpregnant women and 2) the deleterious effect on implantation marked by the NK cytotoxicity assay involves more than lack of inhibitory activity, especially early in the implantation process.

The results of the present study support the concept that immunologic mechanisms are involved in implantation and that NK cells play a role in these mechanisms (2,6–21). In instances where immunologic processes contribute to the lack of success of implantation, appropriate immunotherapy would be expected to enhance implantation rates. However, immunotherapy would not be expected to enhance implantation rates of karyotypically abnormal concepti. Since 60% of preimplantation embryos have been reported to be karyotypically abnormal (4), only 40% of women experiencing implantation failures would be helped if immunotherapy were 100% effective. In our previously reported study (23) intravenous immunoglobulin enhanced successful implantation by 44%. Thus, it is important to have markers to identify IVF failures of chromosomally normal embryos. In addition to risk factors of antiphospholipid antibodies, antinuclear antibodies, elevated circulating NK cells and elevated circulating embryotoxins previously reported (13–17), NK cytotoxicity (17), and elevated circulating levels of CD56+69+ (20) and CD56+16+161+ can be added to markers of risk for immunologic implantation failure. Treatment of implantation failure is dependent on the underlying cause. Identifying the cause is an important component in the successful treatment of implantation failure.

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