Enhancement of peripheral blood CD56dim cell and NK cell cytotoxicity in women with recurrent spontaneous abortion or in vitro fertilization failure

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ABSTRACT

Recent studies support the concept that NK cells play an important role in the success or failure of embryo implantation. Recurrent spontaneous abortion (RSA) is the most common complication of pregnancy. Some couples suffer from infertility of unknown cause. In vitro fertilization (IVF) is one of the useful treatment methods used for treatment of this type for infertility with variable outcomes. The aim of this study was to compare the percentage of peripheral blood CD56− (CD56dim and CD56bright) cells and the level of NK cell cytotoxicity in patients with RSA and patients with IVF failure with those of healthy multiparous and successful IVF control women. In this case–control study peripheral blood samples from 43 patients, which included 23 women with RSA and 20 with IVF failure, plus 43 healthy control women comprising 36 normal multiparous women and 7 women with successful IVF, were collected. The percentage of peripheral blood NK cells (CD56−) was identified by flow cytometry, then peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation (Ficol-Hypaque) and incubated with NK-sensitive K562 cells. The NK cell cytotoxicity level was determined by lactate dehydrogenase (LDH) release assay. The percentage of CD56dim cells and the level of peripheral blood NK cell cytotoxicity in RSA patients and women with IVF failure were significantly higher than in both the healthy multiparous and successful IVF control groups (P < 0.001). The findings of the present study suggest that increases in the percentage of CD56dim cells and NK cytotoxicity in peripheral blood may be important contributing factors for both RSA and IVF failure.

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1. Introduction

Natural killer (NK) cells are the most abundant immune cells infiltrating the uterine implantation site (Moffett-King, 2002). These cells represent the first cellular immune defense mechanism and have close contact with the conceptus and placenta. NK cells comprise 5–12% of all lymphocytes (Thum et al., 2004) and are classified into CD16−CD56dim NK cells and CD16−CD56bright NK cells by their surface markers (Saito et al., 2008). In the peripheral blood, the main population of NK cells consists of CD16−CD56dim NK cells whereas CD16−CD56bright NK cells are the main population in the endometrium (Moffett-King, 2002; Dosiou and Giudice, 2005).

About 0.5–1% of couples who are trying to conceive will suffer subsequent spontaneous abortion (Emmer et al., 2000). Approximately 80% of recurrent spontaneous
miscarriages cannot be accounted for by hormonal disorders, chromosomal deficiencies or uterine abnormalities. Evidence is accumulating that these unexplained miscarriages could have an immunological background (Van Wijk et al., 1996).

The results of the evaluation of different lymphocyte subpopulations in infertility or recurrent miscarriages in the peripheral blood and in endometrial tissues are controversial (Beer et al., 1996; Furuya et al., 2003). Additionally there is no clear cause of at least 20% of infertility cases, and this reproductive failure is also liked with immunological mechanisms (Baczkowski and Kurzawa, 2007).

A previous study showed that an elevated percentage of peripheral blood NK cells is associated with recurrent failed IVF treatment cycles (Beer et al., 1996). A subsequent study showed that increased peripheral blood NK cell cytotoxicity level is also associated with an increased rate of recurrent failed implantation after IVF treatment (Thum et al., 2004).

NK cells play an important role in the cellular recognition and killing of virus-infected and tumor cells (Imai et al., 2000). This process is accomplished through various immune effector mechanisms (Vivier et al., 2008). NK activity is accomplished by nonspecific lysis of infected targets through the use of NK receptors, or the FcγRII (CD16) receptor, which recognizes IgG bound to specific antigens on the target cell surface (Imai et al., 2000). Cytotoxicity assays provide in vitro evaluation of the lytic activity of NK and T cells against tumors or transformed target cells. The evaluation of lytic activity is therefore of great importance in monitoring the functional capability of these cells. CD56 is one of the natural cell adhesion molecule (NCAM) isoforms that are expressed in NK cells. A recent study has suggested that this molecule could be a mediator in the interaction between NK cells and their target cells (Vivier et al., 2008).

The aim of this study was to evaluate the percentage of peripheral blood NK cells (CD56bright and CD56dim) by flow cytometry and then to compare the cytotoxicity activity within peripheral blood NK cells in patients with RSA and IVF, with those of normal multiparous and successful IVF women, using a lactate dehydrogenase (LDH) measurement method.

2. Materials and methods

2.1. Study population

A total of 86 women volunteered to participate in this case-control study. The patient groups comprised 43 women (23 with RSA and 20 with IVF failures). The grade of embryo in the IVF cycles was A or B, where the cleaving embryos were scored according to equality, size of the blastomers and proportion of anucleate fragments. Four categories were distinguished within this scoring system. Type A was defined as an equal sized embryo without anucleate fragments. Type B was defined as a non-equal-sized embryo and a maximum of 20% of the volume of the embryo was filled with anuclear fragments (Staessen et al., 1989).

The control groups included 43 nonpregnant healthy women, which consisted of 36 normal multiparous and 7 women successfully treated with IVF. Blood samples were taken from all women at the secretory phase of the menstrual cycle, which was defined by self-reporting. All persons provided a written informed consent before their participation in the study. Inclusion criteria for women with RSA were a history of at least two consecutive idiopathic miscarriages with the same partner and a desire for pregnancy. A miscarriage was defined as a spontaneous pregnancy loss before 22 weeks of gestation.

The inclusion criteria for IVF failure group were: women who were diagnosed by a gynecologist as having primary or secondary infertility of unknown cause with at least two episodes of IVF failure, no male factor, and with a normal gynecological, hormonal, and anatomical state. While the exclusion criteria included chromosomal rearrangement in either partner, anatomical–hormonal or infectious causes of RSA, immunological disease (presence of antiphospholipid antibodies, lupus anticoagulant, antiphospholipid antibodies), diabetes mellitus, polycystic ovarian syndrome, and thyroid dysfunction, uterine abnormality (fibroid, uterine polyp, uterine septum), normal gynecological, hormonal, and anatomical state. In addition, a history of previous spontaneous abortions, PID, curette, hydrosalpinx, endometriosis, endocrinological, and metabolic diseases, gynecological interventions (leiomyomas, endometrial polyps, and pelvic adhesion removal) were other exclusion criteria. A gynecologist, who is a specialist in infertility, carried out the patient selection carefully. All of the patients selected had a normal karyotype.

The inclusion criteria for control groups were age less than 45 years; normal gynecological, hormonal, and anatomical state; a minimum of one delivery; no history of miscarriage or complications during previous pregnancies. The characteristics of the study population are shown in Table 1.

2.2. Flow cytometric assay

Ten milliliters of peripheral blood was collected in heparinized tubes. The flow cytometry examination was done on the fresh blood. Phycocerythrin (PE)-conjugated antihuman–CD56 monoclonal antibodies (BD Biosciences, USA) were used for NK cell detection. Erythrocytes were lysed using lysing solution (DAKO cytometry, Germany) and washed twice with phosphate buffer saline (PBS). A total of 10,000 events were acquired. CD56+ cell analysis was performed within the lymphocyte cells range. Flow cytometric data were analyzed by WINMDI software.

2.3. Cytotoxicity assay

For the cytotoxicity assay, mononuclear cells (PBMC) were isolated by density gradient centrifugation (Ficoll-Hypaque), washed three times and then resuspended in a freezing medium that included complete medium containing 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen until use. The K562 tumor cell line (National Cell Bank of Iran, Pasteur Institute, Tehran) was maintained in a continuous suspension culture in RPMI 1640 + 10% FCS supplemented with 3 mmol/L L-glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin at 37 °C (Jurisic et al., 1996).
et al., 2001). The K562 cells were used as sensitive target cells for the evaluation of natural killer cell cytotoxicity in vitro (Jurisic et al., 1999; Niu et al., 2001). Determination of natural killer cell function was implemented by an enzymatic colorimetric technique using LDH release (Roche Cytotoxicity Detection Kit plus).

Effector cells were thawed and incubated overnight in medium containing RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine, 100 µg/mL streptomycin and 100 U/mL penicillin. Viability of target and effector cells was determined by the trypan blue dye exclusion test and viability before the cytotoxicity test was >95%. The effector cells at a concentration of 4.0 × 10⁶, 2.0 × 10⁶ and 1.0 × 10⁶ in 100 µL culture medium were mixed with 100 µL of K-562 cells at a concentration of 0.5 × 10⁵, resulting in three effector:target (E:T) ratios: 80:1, 40:1 and 20:1 respectively. Each E:T ratio and control cells were evaluated in triplicate in 96 micro-well plates, incubated at 37°C in a humid atmosphere with 5% CO₂ (Jurisic et al., 1999). The absorbance (LDH activity) was measured in an ELISA reader at 490–630 nm (Tecan-Sunrise Touchscreen, Austria) and the percentage of cytotoxicity was determined using the following equation:

$$\% \text{ of LDH activity} = \frac{\text{ODt} - (\text{ODbg} + \text{ODspont})}{\text{ODmax} - (\text{ODbg} + \text{ODspont})} \times 100$$

where ODt, ODbg, ODspont and ODmax are absorbance readings of the effector cells treated at different concentrations, background (assay solution), spontaneous release from cell culture in RPMI media and maximum readings respectively.

2.4. Data analysis

Data analysis was carried out using SPSS 17.10 software. Comparisons of CD56dim and CD56bright expression and NK cell cytotoxicity between patients and control groups were performed using the Mann–Whitney U test and P < 0.05 was considered significant.

3. Results

The age range of women with RSA and those with IVF failure was 24–42 (median 30 years) and 27–42 (median 31.5 years) respectively. The age range of the normal group (multiparous) was 19–45 (median 30 years) and the age range of successful IVF was 28–34 (median 31.5 years; Table 1).

The median percentage of the CD56dim cell population was significantly higher (P < 0.001) in women with RSA than in multiparous women (12.94% (range: 8.29–23.81) vs. 5.37% (2.17–7.82) respectively). In addition, the result of a comparison of the median percentage in IVF failure and successful IVF (14.08% (8.07–22.05) vs. 5.35% (4.06–7.07) respectively) was significantly higher (P < 0.001) in women with IVF failure. The median percentage of CD56dim in the control groups was 5.35% (2.17–7.82), while in the patient groups it was significantly higher at 13.61% (8.07–23.81) (P < 0.001); these data are shown in Table 2. However, no significant differences were seen in the median percentages of CD56bright cells between any of the patient and control groups (Table 2).

The median percentage of the NK cell cytotoxicity in women with RSA was 32.14% (15.32–76.14), which was significantly higher (P < 0.001) than in normal multiparous women at 10.74% (3.42–18.13). In addition, a significantly higher (P < 0.001) median percentage level of NK cell cytotoxicity was recorded in IVF failure women at 31.3% (17.64–59.12), compared with women after successful IVF at 10.73% (7.89–15.5) (Table 2).

The overall median percentage cytotoxicity levels for both patient groups (RSA and IVF failure) was 32.14% (range: 15.32–76.14), which was also significantly higher (P < 0.001) than the median percentage of the control groups (healthy multiparous and women with successful IVF; 10.7% (3.42–18.13) (Table 2).

4. Discussion

Natural killer cells play an important role in immune responses during pregnancy and changes in the number or in the function of NK cells may be associated with some disorders of pregnancy (Matsumabayashi et al., 2001). The relationship between peripheral blood NK cells and reproductive failure is one of the most controversial areas in reproductive medicine (Chen et al., 2009). Evaluation of peripheral blood NK cells is being promoted as a useful diagnostic test before initiation of a variety of immunosuppressive therapies amongst patients with either recurrent miscarriage or infertility (Rai et al., 2005).

Previous studies have shown poor regulatory NK cells to be associated with certain pathological conditions related...
to pregnancy, for example RSA, infertility with unknown etiology, preeclampsia, endometriosis, and even depression (Dosiou and Giudice, 2005; Kwak-Kim and Gilman Sachs, 2008). In support of this hypothesis, recent studies have focused on NK-cell-related parameters, such as total number of cells, their performance and activities such as cytotoxicity, cytokine secretion, receptors and gene expression in peripheral blood NK cells, endometrium, or decidual NK cells (Kwak-Kim and Gilman Sachs, 2008). Despite these findings, the exact role played by NK cells in pathological conditions related to pregnancy is still controversial. While some believe that immune system suppression is necessary for a successful pregnancy (Beer et al., 1996), and a decrease in the percentage distribution in lymphocytes in peripheral blood or in the uterus has been shown (Baczkowski and Kurzawa, 2007). Increased activity of NK cells has been suspected to be involved in rejection of the fetus by the prevention of the trophoblast invasion of the endometrium (Lunghi et al., 2007), while others disagree (Beer and Kwak, 1998).

Natural killer cells are an abundant population of human lymphocyte in the decidua, and are therefore expected to play a physiological role during the implantation process (Kwak et al., 1995; Matsubayashi et al., 2005). However, despite many similarities between the two groups of NK cells in peripheral blood and decidua, they are some differences. These differences prompted the renaming of the NK cells that exist in decidual uNK cells (Zhang et al., 2005). It is interesting to consider that uNK cells are found in large numbers at the implantation site where they come into close contact with trophoblast cells (Moffett et al., 2004). Trophoblasts are also in contact with maternal blood where the NK population is primarily CD56dim CD16+. The majority of peripheral blood NK cells are CD56dim CD16+, but most of the uNK cells are CD56bright CD16+ (Dosiou and Giudice, 2005). In addition, trophoblast cells express antigens shared with the tumor cells and viruses that do not express HLA1 and HLA2 and are not killed by uNK cells. Indeed, the mechanism by which trophoblast cells escape from uNK cells is unclear. However, it is hypothesized that enhancement of the number of NK activities can lead to trophoblast cell killing by these cells and may lead to abortion (Kwak-Kim and Gilman Sachs, 2008).

Despite the mismatch between the peripheral blood NK cells and uNK cells, many studies in this field are based on peripheral blood NK cells. In order to gain a better view of the role of uNK, some studies have used biopsies taken from the endometrium of RSA patients (Lachapelle et al., 1996). In our study patients did not agree to provide endometrial specimens; thus, we used peripheral blood.

There is evidence to show that the percentage of CD56+ NK cells is significantly reduced in peripheral blood in the first trimester of a normal pregnancy (Kwak et al., 1995). This finding is compatible with the idea that, in normal pregnancy, the immune system is suppressed. However, there are no reports on the precise amount of peripheral blood CD56dim NK cells that are reduced during this period. Previous studies on the percentage change in CD56dim or CD56bright had different results. There were no statistical differences in the absolute count of CD56dim CD16+ NK cells and CD56bright CD16+ NK cells between successful and failed IVF treatment. However, there was an increase in the absolute count in activated NK (CD56dim CD16+ CD69+) cells in peripheral blood that was found to be associated with a reduction in the rate of implantation in women undergoing IVF treatment (Thum et al., 2004). The same authors, in another study, reported that there were no significant differences between simple enumerations of peripheral blood NK cells (including total CD56+ NK, CD56dim NK and CD56bright NK cells) in IVF treatment and pregnancy outcome (Thum et al., 2005). On the other hand, the percentages of CD56bright CD16− NK cells were higher in the control group than in both ICSI-treated groups (Baczkowski and Kurzawa, 2007). However, there is no general consensus on the effect of dim or bright NK cells.

The results from the present study showed no significant differences in the percentage of CD56bright among women with RSA and those with IFV failure compared with the control groups. These findings are in agreement with those of Thum et al. (2004). However, it is possible that due to the low percentage of the CD56bright cell population, changes in the number of these cells between the patient and control groups may not have been accurately

Table 2
Comparison of the median percentage of CD56dim, CD56bright cell population and NK cytotoxicity among normal, RSA, successful IVF, IVF failure, total control and total patient groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>Median % CD56dim (range)</th>
<th>Median % CD56bright (range)</th>
<th>Median % cytotoxicity (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>36</td>
<td>5.37 (2.17–7.82)</td>
<td>0.62 (0.12–0.92)</td>
<td>10.74 (3.42–18.13)</td>
</tr>
<tr>
<td>RSA</td>
<td>23</td>
<td>12.94* (8.29–23.81)</td>
<td>0.61 (0.1–1.5)</td>
<td>32.14* (15.32–76.14)</td>
</tr>
<tr>
<td>Successful IVF</td>
<td>7</td>
<td>5.35 (4.06–7.07)</td>
<td>0.65 (0.35–0.95)</td>
<td>10.73 (7.89–15.5)</td>
</tr>
<tr>
<td>IVF failure</td>
<td>20</td>
<td>14.08* (8.07–22.05)</td>
<td>0.57 (0.28–1.02)</td>
<td>31.3* (17.64–59.12)</td>
</tr>
<tr>
<td>Total control</td>
<td>43</td>
<td>5.35 (2.17–7.82)</td>
<td>0.63 (0.12–0.95)</td>
<td>10.7 (3.42–18.13)</td>
</tr>
<tr>
<td>Total patients</td>
<td>43</td>
<td>13.61* (8.07–23.81)</td>
<td>0.61 (0.1–1.5)</td>
<td>32.14* (15.32–76.14)</td>
</tr>
</tbody>
</table>

* P<0.001 between RSA and normal.
* P<0.001 between successful IVF and IVF failure.
* P<0.001 between total control and total patients (Mann–Whitney U test).
measured and should be reviewed in a wider population. In contrast, the percentage of CD56\textsuperscript{dim} cells was higher in women with IVF failure and in those with RSA in comparison to their control groups. An abnormal enhancement in peripheral blood-activated CD56\textsuperscript{dim} NK cell parameters related to infertility with unknown causes has been shown in a previous study (Thum et al., 2004).

The effect of freezing on cell activity remained relatively stable up to 14 months of storage, which confirms that freezing damage depends on the freezing conditions rather than on the duration of cryopreservation (Saeko et al., 1986) and that DMSO is a suitable medium for freezing (Alsayed et al., 2008). In the present study we employed freezing using DMSO. However, it is expected that small changes in cell activity may have occurred, but since both patients and normal controls underwent similar freezing processing conditions, it is presumed that the cryopreservation has not had an effect on our results.

The results from different studies on NK cytotoxicity are controversial. For example, Souza et al. (2001), using Cr-release assay, reported that peripheral blood NK cell cytotoxicity did not differ between patient and control groups. In contrast to this study, our results showed a clear and significant increase in NK cytotoxicity among RSA women in comparison with normal women. These results are in agreement with Yamada et al. (2003), using both 51Cr release and flow cytometric analysis. One reason for the controversy regarding the results obtained from different studies may be due to sampling time taken from patients with RSA and those with IVF failure. Therefore, in order to assess the significance of this factor, it may be better to compare the cytotoxicity among RSA patients during pregnancy and after their abortion. This question can be answered in pregnant women with IVF failure, both during pregnancy and after losing their pregnancy in future studies.

Most previous studies considered women with RSA or those with IVF failure singly, while in our study women with RSA those with IVF failure were studied simultaneously. In the current study, we first assayed the percentage of CD56\textsuperscript{dim} and CD56\textsuperscript{bright} cells and then NK cytotoxicity. It is interesting that the results of CD56\textsuperscript{dim} and NK cytotoxicity were in agreement with each other and both were increased in women with IVF failure and those with RSA in comparison with their control groups. Because CD56\textsuperscript{dim} cells have more cytotoxic activity than CD56\textsuperscript{bright} cells, it would seem logical that with the increase in the population of these cells, NK cytotoxicity should also increase. On the other hand CD56\textsuperscript{dim}, as the major peripheral blood NK cells, have close contact with choric villi at the intervillous space (Chen et al., 2009) and their increase together with NK cell cytotoxicity may be two reasons for unsuccessful pregnancies in women with IVF failure and those with RSA.

In this study, the LDH method was used for NK cell cytotoxicity determination, whereas in the majority of the other studies either 51Cr-release assay, flow cytometry, or MTT quantification were used. A study in which the accuracy of the LDH method was compared with Cr-release assay for NK cytotoxicity showed that these assay results were comparable (Konjevi et al., 2001). Cr-release assay is an accurate test for cytotoxicity evaluation, but cytotoxicity assay using the LDH method is also easy and reliable. Furthermore, the advantage of the latter method is that it does not need radioactive material, thus avoiding exposure to radiation.

In conclusion, this study indicates that NK cell cytotoxicity and the percentage of CD56\textsuperscript{dim} cells in patients with RSA and those with IVF failure were significantly higher than in control samples and is in agreement with other studies (Emmer et al., 2000; Kwak et al., 1995; Thum et al., 2004). Therefore, an increase in peripheral blood NK cell cytotoxicity and CD56\textsuperscript{dim} cells in women with RSA and those with IVF failure may be an important factor in the inability to have a successful pregnancy.

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