Analysis of cytotoxic activity of peripheral blood natural killer cells in women with recurrent miscarriage

Abstract

Around 50% of recurrent spontaneous abortions (RSA) remain unexplained. Immunological etiology has been proposed, supported by evidence of lower count of natural killer (NK) cells in peripheral blood of RSA women compared to women with normal delivery history. However, studies concerning the cytotoxic activity of NK cells in women with RSA are still controversial. We performed an observational case-control study assaying peripheral blood NK (pNK) cells cytotoxic activity in non-pregnant RSA women compared to non-pregnant women with normal delivery history. Twelve RSA and nine control women were recruited and blood samples were drawn during the luteal phase of ovarian cycle. pNK cells were incubated with target CFSE-labeled K562 cells and cytotoxicity was measured by cytofluorimetry. In non-pregnant RSA women pNK cytotoxic activity was not significantly altered compared to control women. In the luteal phase of ovarian cycle the level of cytotoxic activity of pNK cells is not a marker for predicting RSA, and clinicians should not use pNK activity as a systematic recurrent pregnancy loss examination.

Keywords: spontaneous abortion, recurrent miscarriage, natural killer cells, cytotoxicity, cytofluorimetry

Introduction

Although at least one of these causes is present in about half of patients, in the other half RSA remains completely unexplained. Around 1% of couples who are trying to conceive will suffer recurrent spontaneous abortion (RSA), defined as three or more subsequent pregnancy loss before 20 weeks of gestation. Several causes have been proposed for RSA, including chromosomal abnormalities, antiphospholipid syndrome, uterine malformations, endocrine defects, and cervical incompetence.

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pNK cells play a central role in the early defenses against viral infection acting through cytolytic activity secretion of pro-inflammatory cytokines. Around 90% of peripheral blood NK (pNK) cells are CD16+ CD56dim and have high cytotoxic activity, while the CD16-CD56 bright subgroup is the main source of NK cell-derived immuno-regulatory cytokines. UnNK cells derive from this latter subpopulation, promote placental and trophoblast growth, and provide immunomodulation at the maternal-fetal interface. Evidence suggests that NK cell number and cytotoxicity level are hormonally regulated. During the luteal phase of the ovarian cycle uNK number, pNK cell number and cytotoxicity are increased compared to the follicular phase. In normal human early pregnancy, progesterone causes decreasing in pNK cell number and activity, and recruitment of uNK cell by cytokines secreted from endometrial stromal cells, which also regulate uNK cell proliferation and differentiation, ultimately supporting local immunomodulation and placenta development. Dysregulation of NK cells has been hypothesized to play a role in RSA, as initially suggested from evidence of increased number of pNK cells in women with RSA history compared to control women and more recently a large cohort study in Japan reported no association between pNK cytotoxic activity and risk of subsequent miscarriage in women with RSA.

pNK cell number and pNK cell cytotoxicity do not strictly correlate, as it is possible to have significant pNK activity even in presence of low pNK cell count. As regards to the uterine compartment, it was hypothesized that the activity level of unNK cells present in the lining of womb during early pregnancy could influence the embryo survival, rather than their absolute cell count. It is not clear, however, if this applies also to pNK cells, i.e. if activity level of pNK cell associates with risk of spontaneous abortion.

The aim of the present observational case-control study is to investigate if the level of pNK cytotoxic activity associates with RSA in Italian population, comparing a group of RSA women with a group of matched control women with normal pregnancy history.

Materials and methods

Study subjects were recruited at the Gynecology and Obstetrics Units of the S. Anna University Hospital of Ferrara, Italy, during year 2014. A total of 12 women with history of at least two

Abbreviations: RSA, recurrent spontaneous abortions; pNK, peripheral blood natural killer cells; uNK, uterine natural killer cells; BMI, body mass index
idiopathic spontaneous miscarriage with the same partner and a
desire for pregnancy were selected. A miscarriage was defined as a
spontaneous pregnancy loss before 22 weeks of gestation. Women with
recurrent abortions presumably related to specific pathologies,
such as immunological diseases, uterine malformation or hormonal
dysfunction, were excluded from the study. Age, body mass index
(BMI) and obstetrical history of the women included in the study
are reported in Table 1. A control group of 9 women with a history
of normal pregnancy and delivery was recruited in the same period.
This study was approved by Local Ethical Committee (CE-BIF
24.10.2013), and all subjects included in the study provided a written
informed consent before their participation, in agreement with the
World Medical Association Declaration of Helsinki.

Table 1 Age, BMI; and obstetrical history of women with idiopathic spontaneous abortion included in the study

| Mean ± SD | Range |
|-----------|-------|
| Age ‡ | 35.5 ± 4.3 | 29-45 |
| BMI § | 23.8 ± 5.6 | 18.5-34.0 |
| N° SA ¶ | 3.3 ± 3.0 | 2-6 |
| Parity | 0.2 ± 0.4 | 0-1 |

†, standard deviation; ‡, age in years; §, BMI: body mass index; ¶SA, number spontaneous abortion events.

From all participating subjects a peripheral blood sample was drawn
during the luteal phase of ovarian cycle, as assessed by a
gynecology specialist. Peripheral blood mononuclear cells (PBMCs)
were isolated by density centrifugation. pNK cells cytotoxicity
was estimated after co-culturing PBMC with carboxyfluorescein
cucinidimyl ester (CSFE)-labelled K562 target cells, and detecting
cell death by propidium iodide (PI) staining using flow-cytometry. K562
cells are from erythromyelocytic leukemia, are specifically
killed by NK cells but not by other cells. K562 cells were maintained
in RPMI 1640 with L-glutamine and NaHCO₃ (Sigma-Aldrich, St.
Louis, USA), supplemented with 10% Fetal Bovine Serum (Gibco/
Invitrogen, Karlsruhe, Germany), 100IU/ml penicillin and 100µl/ml
streptomycin (Gibco/Invitrogen, Karlsruhe, Germany) at 5% CO₂,
37°C. One day prior to cytotoxic assay cells were sub-passaged
to ensure growth in the log phase. After two washing with cold
phosphate buffered solution (PBS), K562 cells were resuspended
at 5x10⁶ cells/ml concentration in pre-warmed PBS (37°C), and
labeled with 0.1µM CSFE (CellTraceTM CSFE Cell Proliferation
kit, Molecular probes/Invitrogen, Eugene, Oregon) for 10min at
room temperature in the dark. CSFE penetrates plasma membranes
and covalently binds cytoplasmic proteins, yielding green fluorescent
target cells. The reaction was stopped by adding 4-5 volumes of RPMI
1640 containing 10% FBS and incubating on ice for 5 minutes. CSFE-
labelled K562 cells were washed three times with RPMI 1640 with
10% FBS and resuspended at the final concentration of 1x10⁶
cells/ml. Cell viability, as assessed by Trypan Blue dye exclusion assay,
was always over 95%.

Effector cells (PBMCs) were isolated from 5ml of heparinized
peripheral blood samples by density gradient centrifugation on
Lymphocyte (Cederlane, Burlington, 22 weeks of gestation). PBMC were
washed twice with RPMI 1640 and resuspended at 5x10⁶ cells/ml in
RPMI 1640 containing 10% FBS.

PBMCs and CSFE-labelled K562 cells were co-incubated in
flat bottom 48-well sterile polystyrene plates (Costar corporation,
Cambridge, Massachusetts, USA) at an effector/target (E:T) ratio
of 10:1. Cultures were incubated at 37°C in 5% CO2 atmosphere
for 4 hours. Target cells were assayed separately to determine the
background level of spontaneous cell death. As positive control
reference, the maximum NK activity was obtained by stimulating
effector cells with 2.5µg/ml phorbol 12-myristate 13-acetate (PMA,
Sigma-Aldrich, St. Louis, USA) and 0.5µg/ml Ionomycin (Sigma-
Aldrich, St. Louis, USA) one hour prior to adding target cells.

After co-incubation, cells were treated with PI (Sigma-Aldrich, St.
Louis, USA) at final concentration of 1µg/ml to label dead target cells.
After 15 min of incubation on ice in dark, samples were analyzed
using BD FACS CALIBUR flow cytometer and CellQuest Software.
The percentage of cytolytic activity was determined by gating the target cells population in a FL1
dot plot (CSFE green fluorescence) versus FL3 (PI red fluorescence).
Data for at least 2000 target cells were analyzed for each test.

The cell-mediated cytolytic activity of pNK cells was expressed
as percentage of dead target cells. Each experiment was done in
duplicate, and background values subtracted. Results were expressed
as average values and 95% confidence interval after normalization
to PMA/Ionomycin reference. Comparison of results obtained in RSA
and control women was done by “two sample t-test between percent”
using Statistic Calculator software version 4.0 (StatPac Inc., Pepin,
WI, USA).

Results and discussion

In assays carried using PBMC from RSA or control women the
stimulation with PMA/Ionomycin induced cytolysis of 55.6%. This
value can be taken as the maximal pNK cytolytic activity of the
assay. Cytotoxic activity of NK from PBMCs from RSA patients or
controls using K562 as target is reported in Figure 1A. Among the BD
controls used in the study mean value of pNK cell cytotoxic
activity was 3.73% (95% CI 2.94-4.52) (Figure 1B), while resulted
on average of 4.30% (95% CI 3.44-5.17) among the 9 control women
(Figure 1C). The two mean values resulted not significantly different,
as case-control comparison using “two sample t-test between
percent” turned out with t-statistic of 0.067 and a P-value of 0.947.
Moreover, no association was observed between age and pNK cells
cytotoxic activity among RSA patients, nor among control women
(data not shown).

NK cells are the most abundant population of lymphocyte present
at maternal-fetal level and play a fundamental role in implantation
process and in pregnancy outcome. Increased activity of NK
cells has been suspected to be involved in rejection of the fetus by
preventing trophoblast invasion of the endometrium. Several studies
investigated alteration of NK cells cytotoxicity or cell number
as cause of RSA or other pregnancy-related pathological conditions,
but results are still controversial. Some investigations, focusing on
uNK, report no difference in total uNK number in women with RSA
compared with women with normal delivery history, although a
trend for a decrease number of CD16-CD56 bright NK cell subset
compared to the number of CD16+CD56dim NK cells was observed. This
effect could be related to an increase in NK number or activity in
peripheral blood, causing a higher recruitment of CD16+CD56bright
NK cell in the endometrium.

In the present study, based on PI staining of CSFE-labelled K562
target cells and cytofluorimetric detection, no significant difference in

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pNK cells cytotoxic activity was found in RSA compared to control women. This result is in agreement with Souza et al.,16 that used a chromium-51 release cytotoxicity assay and reported no difference in pNK cell cytotoxicity between a group of 9 RSA patients and a control group of 9 women, and with a Dutch study based on 43 RSA patients and 37 controls.24 Our results also replicate well the findings of Nagoya’s study that used the same chromium-51 release cytotoxicity assay and included a large cohort of 552 RSA cases.19 However, a study carried out in Iran on 23 RSA cases and 36 controls but based on lactate dehydrogenase (LDH) release assay resulted with significantly higher pNK cytotoxic activity among RSA women compared to controls, along with an increased number of CD56dim cells.15 In this latter study the secretory phase of ovarian cycle of women included in the study was defined by self-reporting instead of determined by a gynecologist, and this could have influenced the study outcomes, but we cannot exclude that different results could be consequence of the diverse method used to assess pNK cytotoxic activity. Moreover, we cannot ignore that controversial results across studies could rely on differences in the environmental conditions to which different studied populations are exposed to, or to different genetic background.

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Conflict of interest

The author declares that there are no conflicts of interest.

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