Relationship between uterine natural killer cells and unexplained repeated miscarriage

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Abstract

Objective: To evaluate the relation between uterine killer (uK) cells and unexplained repeated miscarriage (RM).

Material and Methods: Eighty women with unexplained repeated miscarriage and missed miscarriage of current pregnancy were studied. Fetal viability and gestational age of the current pregnancy were confirmed by ultrasound, followed by suction evacuation to collect abortion specimens and uterine wall curettage to collect decidua specimens. Abortion specimens were collected for long-term monolayer cell culture and subsequent chromosome analysis using conventional G-banding. Decidua specimens were subjected to immunohistochemical staining using monoclonal antibodies specific to CD56+ and CD16+ expressed by uK cells.

Results: CD56+ CD16+ uK cells were found in 85% [68/80] of the studied decidua specimens of women with unexplained repeated miscarriage; 88.5% [54/61] had normal abortion karyotyping and 73.7% [14/19] had abnormal abortion karyotyping.

Moreover, 73.75% [59/80] of the studied women with a past history of early miscarriage had CD56+ CD16+ uK cells in their decidua specimens, and 66.25% [53/80] of studied women with a past history of late miscarriage had CD56+ CD16+ uK cells in their decidua specimens; the association between early and late miscarriage and CD56+ CD16+ uK cells in decidua specimens was significant.

Conclusion: CD56+CD16+ uK cells were predominant in the decidua specimens of the studied women with repeated miscarriage. A significant association was found between the presence of CD56+ CD16+ uK cells in the studied decidua specimens and unexplained repeated miscarriage.

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Introduction

Repeated miscarriage (RM) is defined as two or more failed pregnancies (confirmed by ultrasound or histopathological examination) and is known to affect approximately 0.5–1% of couples (1).

One miscarriage increases the risk of miscarriage in future pregnancy to 24%; this risk increases to 26% with two previous miscarriages and reaches 32% with three previous miscarriages; thus, women with two or more consecutive miscarriages merit meticulous study to detect the definite cause and possible treatment (2-4).

Various factors are implicated in the pathophysiology of repeated miscarriage. Fetal causes such as single gene or genomic imprinting defects account for 3.5–5% of the cases of repeated miscarriage; other fetal defects include fetal infections and developmental abnormalities (5). Maternal causes of repeated miscarriage include immunological causes, accounting for 30% of the cases, with anti-phospholipid antibody syndrome being the most common autoimmune cause (6, 7). Endocrine dysfunction accounts for 48.71% of the cases, while other maternal factors, including anatomical detects and subclinical endometrial infection, account for a minimal number of cases (8, 9).

Approximately 50% of repeated miscarriages are unexplained, with no definitive etiology. Several authors suggest the cause to be alloimmune rejection of the fetus (10).

Natural killer (NK) cells are immune system lymphocytes (11, 12). Uterine killer (uK) cells are short-lived lymphocytes found in uterine deciduas (13). Early in pregnancies, uK cells produce angiogenic factors and are believed to be important for implantation and development (13, 14).

Uterine killer cells have been linked to human reproductive disorders, including repeated miscarriage, implantation failure, fetal growth restriction, and preeclampsia (15, 16). These cells secrete cytokines and angiogenic factors, which are important for placental development and pregnancy establishment (16).

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It has been found that 37.3% of patients who presented with repeated miscarriage had a mild to moderate increase in NK cells and that 14.7 % of women with repeated miscarriage had elevated levels of $CD56^+$ NK cells in peripheral blood (17, 18).
Other authors concluded that the cytotoxicity of NK cells is unrelated to the number of peripheral NK cells and that it can be estimated by NK cell markers such as killer inhibitory receptors (KIRs) or $CD16^+$ receptor expression (19).
Because more research is needed to establish the relationship between uK cells and human reproductive disorders (20, 21), this study was designed to evaluate the relationship between uK cells and unexplained repeated miscarriage.

Material and Methods

Eighty women with unexplained repeated miscarriage and missed miscarriage of current pregnancy were included for evacuation and curettage because of a current missed miscarriage (>8 weeks, diagnosed by ultrasound). The women were studied after proper counseling, consent, and approval of the ethical committee. Unexplained repeated miscarriage was defined as ≥2 previous miscarriages after <20 weeks' gestation. A thorough history was obtained and a thorough examination was performed for all studied women, followed by trans-vaginal ultrasound to confirm fetal the viability and gestational age of the current pregnancy by a sonographer who was blinded to the patients' data. Peripheral venous samples were collected from the studied women for oral glucose tolerance tests; thyroid stimulating hormone assays; prolactin, serum anticardiolipin, and lupus anticoagulant assays; as well as activated protein C resistance tests, Leiden factor V and prothrombin gene mutations, and protein C, S, and anti-thrombin III deficiency tests. Women with septic miscarriage, documented endocrinopathies (diabetes, thyroid disorders, or hyperprolactinemia), uterine anomalies, polycystic ovary syndrome, anti-phospholipid antibody syndrome, thrombophilia, abnormal karyotype in one or both parents determined by leukocyte culture, autoimmune disorders, history of hormonal contraception, and a history of intrauterine contraceptive device application within the last three months preceding current pregnancy were excluded from this study. Evacuation and curettage were performed for all women included in this study under general anesthesia using suction evacuation to collect abortion specimens after cervical dilatation, followed by uterine wall curettage to collect decidua specimens. Abortion specimens were collected in a special medium for long-term monolayer cell culture and subsequent chromosome analysis using conventional G-banding. Decidua specimens were subjected to immunohistochemical (IHC) staining using monoclonal antibodies specific to uK cells $CD56^+$ and $CD16^+$.

Reagents and materials used include the following:

1. Primary antibodies: Liquid monoclonal mouse antibody (MoAb) against $CD56^+$ and $CD16^+$ expressed on NK cells.
2. Universal Kits: Supersensitive immunodetection system (Biogenex Laboratories; San Francisco, USA), containing the following: a) negative control antibody; b) biotinylated anti-immunoglobulin for mouse antibody; c) label: streptavidine peroxidase complex; d) chromogen: 2.3 diaminobenzidine chromogen (DAB) solution, ready to use substrate buffer, and H_2O_2 substrate for use with liquid DAB chromogen and substrate buffer; e) blocking reagent to block endogenous peroxidase activity.
3. Lyophilized pepsin powder, phosphate buffer saline (PBS), counter stain (Mayer's hematoxylin), distilled water, and mounting media (Canada balsam).
4. Staining jars, microscopic positive charged slides, cover slips for slides, and immune-stainer.
5. Light microscope with 100x and 400x magnification.

IHC procedure: Decidua specimens were fixed in buffered formalin (not more than 24 hours) and embedded in paraffin wax; 3-micometer sections were mounted onto 3-aminopropyltriethoxysilane, (Sigma Chemical Co.; Poole, UK); then serial sections were stained for uK cells ($CD56^+$ and $CD16^+$) using antibody antigen-retrieval methods (22, 23).
Primary antibodies were incubated for 60 minutes for $CD56^+$ and for 120 minutes for $CD16^+$ at room temperature; the brown staining intensity of the reaction developed with 2.3 DAB containing 0.01% H_2O_2 was noted, and the sections were counterstained with hematoxylin, then dehydrated and mounted with distyrene, plasticizer, xylene (DPX) standard resin (Lamb Ltd.; London, UK), then examined by ordinary light microscopy. Appropriate positive controls (neuroblastoma for $CD56^+$ and tonsils for $CD16^+$) were used in each run to judge the effectiveness of the staining technique, and mouse immunoglobulin-G (Ig-G) antibodies were used instead of primary antibodies as negative controls.

Sample size and statistical analysis

The required sample size to produce statistically acceptable figure was 80 women, and this sample size was calculated using G Power software (Heinrich Heine Universität; Düsseldorf, Germany) for sample size calculation. Mean±SD was used to represent numerical values, while number (n) and percentage (%) were used to represent categorical values. Comparison between the variables was done using the Chi-square (x^2) test. A difference with $p<0.05$ was considered statistically significant.

Results

The mean age of the women included in this study was 29.6±6.39 years and the mean body mass index (BMI) was 26.9±4.5 kg/m^2. Karyotyping studies of the abortion specimens showed normal karyotyping in 76.25% [61/80] of the studied specimens and abnormal karyotyping in 23.75% [19/80] of the studied specimens (Table 1).
$CD56^+$ $CD16^+$ uK cells were found in 85% [68/80] of the studied decidua specimens of women with unexplained repeated miscarriage; 88.5% [54/61] had normal abortion karyotyping and 73.7% [14/19] had abnormal abortion karyotyping (Table 2).
73.75% [59/80] of the studied women with a past history of early miscarriage had $CD56^+$ $CD16^+$ uK cells in their decidua specimens and 66.25% [53/80] of the studied women with a past history of late miscarriage had $CD56^+$ $CD16^+$ uK cells in their decidua specimens; the association between early and late miscarriage and $CD56^+$ $CD16^+$ uK cells in the decidua specimens was significant (Table 3).

Table 1. Karyotyping analysis of the studied specimens

Variable	Number (n)	Percentage (%)
Normal female karyotype	58	72.5
Normal male karyotype	3	3.75
Abnormal karyotype	19	23.75
Triploidy	5	6.25
Tetraploidy	10	12.5
Aneuploidy	4	5.0
Total	80	100

Table 2. Relationship between karyotypes of abortion specimens and immunohistochemical results of decidua specimens

Variable	$CD56^+$ $CD16^+$ uterine killer cells n (%)
Normal karyotype (61 cases)	54 (88.5%)
Abnormal karyotype (19 cases)	14 (73.7%)
Total	68 (85%)

CD: classification determinant
CD receptors expressed on uterine killer cells.

Table 3. Relationship between CD56+ CD16+ uterine killer cells and miscarriage (early and late)

Variables	$CD56^+$ $CD16^+$ uterine killer cells n (%)
Number of early miscarriages	
1	13 (16.25%)
2	25 (31.25%)
3	12 (15%)
>3	9 (11.25%)
Total (80 cases)	59 (73.75%)
Number of late miscarriages	
0	4 (5%)
1-2	53 (66.25%)
Total (80 cases)	57 (71.25%)

CD: classification determinant
CD: receptors expressed on uterine killer cells.

Discussion

Early in pregnancies, uK cells produce angiogenic factors and are believed to be important for implantation and development. uK cells have been linked to human reproductive disorders, including repeated miscarriage, repeated implantation failure, fetal growth restriction and preeclampsia (15, 16); this study was designed to evaluate the relationship between uK cells and unexplained repeated miscarriage.

CD16 is expressed by most natural killer cells, neutrophils, and activated macrophages. CD56 is an isoform of the neural cell adhesion molecule and is expressed on natural killer cells, cytotoxic T lymphocytes, and neural-derived cells. Peripheral natural killer (pNK) cells have been found in both peripheral blood and endometrium. Although both pNK and uterine natural killer (uNK) cells express the surface antigen CD56, they are phenotypically and functionally different (11). Studies have shown that 90% of pNK cells express a $CD56^{dim}$ $CD16^+$ phenotype, while 80% of uNK cells express a $CD56^{bright}$ $CD16^-$ phenotype; the CD56 cells are known to have a regulatory function, while the CD16 cells have a cytotoxic function (24-26).

In humans, it has been proved that elevated circulating cytotoxic NK cells (not the count) increase the risk of miscarriage (27). Women <35 years old with unexplained repeated miscarriage were studied to minimize the risk of chromosomal abnormalities and miscarriages associated with advanced maternal age (28).

The BMI of women included in this study was 26.9±4.5 kg/m^2; this might be due to our selection criteria, as we excluded some risk factors that might predispose the women to RM, such as obesity (29, 30), diabetes mellitus, and thyroid disorders. Eighty abortion specimens were cytogenetically analyzed using tissue culture and conventional G-banding because comparative genomic hybridization (without culture) was not introduced in our institute until recently. Cytogenetic analysis using tissue culture and conventional G-banding has some limitations, including contamination, culture failure, and maternal cell growth (31). In this study, karyotyping studies showed normal karyotyping in 76.25% [61/80] of the studied abortion specimens and abnormal karyotyping in 23.75% [19/80] of the studied abortion specimens. A 29 to 57% rate of chromosomal abnormality was previously reported during analysis of miscarried tissue from women suffering RM (32-35), and the higher number of normal chromosomes studied in the miscarried tissue of women with RM confirms that there may be factors other than chromosomal abnormalities associated with RM (36).

$CD56^+$ $CD16^+$ uNK cells were found in 85% [68/80] of the studied decidua specimens of women with unexplained repeated miscarriage. Quenby et al. (16) reported that women with RM had significantly more uNK than controls, and Clifford et al. (37) also showed increased CD56+ uK cells in women with unexplained repeated miscarriage.

Increased expression of $CD56^+$ $CD16^+$ uNK was also reported in deciduas obtained after spontaneous miscarriage in women with a history of repeated miscarriage (38).

Quenby et al. (39) used IHC to investigate leukocyte populations in mid-luteal endometrial biopsies of 22 women suffering from RM compared to 9 women without RM, they found that $CD4^+$, $CD14^+$, $CD16^+$, and $CD56^+$ uNK cells were significantly higher in the RM group than in controls.

Lachapelle et al. (40) compared endometrial specimens from 20 women with RM with endometrial samples collected during the secretory phase from 15 fertile controls. Lachapelle et al. (40) found that the percentage of uK was similar in the two groups, although a greater percentage of $CD56^+$ $CD16^+$ uK was found in women with RM.

$CD56^+$ $CD16^+$ uNK cells were found in 85% [68/80] of the studied decidua specimens of women with unexplained repeated

miscarriage; 88.5% [54/61] had normal abortion karyotyping and 73.7% [14/19] had abnormal abortion karyotyping. This difference was statistically not-significant. Yamamoto et al. (41) reported the same findings when they studied uNK cells in decidua specimens of both chromosomally normal and abnormal missed miscarriages.

Although in this study we found that the expression of $CD56^+$ $CD16^+$ uNK cells in decidua specimens of women with RM is high, Yamamoto et al. (41) did not find overexpression of $CD56^+$ $CD16^+$ uK cells in the deciduas of studied women with missed abortions, because their study was limited to sporadic cases of missed abortions, not RM cases.

73.75% [59/80] of studied women with a past history of early miscarriage had $CD56^+$ $CD16^+$ uK cells in their decidua specimens and 66.25% [53/80] of studied women with a past history of late miscarriage had $CD56^+$ $CD16^+$ uK cells in their decidua specimens; the association between early and late miscarriage and $CD56^+$ $CD16^+$ uK cells in the decidua specimens was significant. The findings of this study suggest that $CD56^+CD16^+$ uK cells are predominant in the decidua of women with RM. Women refused to participate in the study and the use of tissue culture and conventional G-banding for cytogenic analysis and karyotyping of abortion specimens were limitations during this study. Further large case-controlled studies are needed to compare decidua specimens from RM cases with decidua specimens from normal cases without RM to establish the relationship between uK cells and human reproductive disorders and to improve future treatment for such cases.

Ethics Committee Approval: *Ethics committee approval was received for this study from the local ethics committee of Ain Shams University, Maternity Hospital.*

Informed Consent: *Written informed consent was obtained from patients who participated in this study.*

Peer-review: *Externally peer-reviewed.*

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