Original Article

Uterine CD56^{dim} and CD16^{+} Cells in Refractory Antiphospholipid Antibody-Related Pregnancy Loss and Chromosomally Intact Abortuses: A Case–Control Study

Mostafa F. Gomaa, Abdeiiatif G. Elkhouly, Mohammad M. Farghly, Laila A. Farid, Nahla M. Awad[1]

Department of Obstetrics and Gynecology, Ain Shams University, Cairo, Egypt, [1]Consultant Pathology, Early Cancer Detection Unit, Ain Shams University, Cairo, Egypt

Abstract

Aim: To evaluate the role of uterine natural killer (uNK) CD56^{dim} and CD16^{+} cells in patients with refractory antiphospholipid, antibody-mediated, recurrent, pregnancy loss. **Settings and Design:** A case–control study was conducted between 2012 and 2015 at a university hospital. **Patients and Methods:** A group of 118 women with a history of antiphospholipid antibody syndrome experiencing fetal loss in spite of low dose aspirin (LDA) and low molecular weight heparin (LMWH) treatment in the current pregnancy were included in this study. A group of 32 patients undergoing an elective termination of viable pregnancies before 20 weeks were taken as controls. Suction evacuation was performed to collect abortus specimens, and uterine wall curettage was performed to collect decidua specimens, which were then stained using monoclonal antibodies specific to CD56 and CD16. **Statistics:** Statistical analyses were performed using the Statistical Package for the Social Sciences version 18 software. Chi-square and Fisher exact tests were used for making comparison between the groups. **Results:** Abnormal fetal karyotype was found in nine (9/97) cases of the study group, which means that abnormal karyotype accounts for only 9.3% of the causes of failure of treatment. Abnormal karyotype was found in four cases of the control group. Only cases with normal karyotyping were subjected to decidual uNK cells analysis. We found that CD56^{dim} and CD16^{+} were found in the decidua of 79 cases (79/97), which means that aberrant natural killer cells expression might account for 81.4% of the cases of refractory antiphospholipid antibody (APA)-mediated recurrent pregnancy loss. **Conclusion:** CD56^{dim} and CD16^{+}uNK cells might be correlated with refractory APA-mediated recurrent pregnancy loss.

Keywords: *Antiphospholipid antibodies, recurrent miscarriage, uNK, uterine natural killer cells*

Introduction

Recurrent spontaneous abortion (RSA) refers to the loss of pregnancy for ≥2 times in patients before the 20 weeks of gestation or a fetal weight of <500 g.[1,2] Although it has been estimated to affect 5% of women in the reproductive age, it is believed that as per these statistics, the incidence of RSA is even higher because of the exclusion of subclinical and under diagnosed RSAs.[3]

The etiology of RSA includes chromosomal, anatomic, endocrine, and autoimmune abnormalities, as well as the infections of the reproductive tract. In at least 35–44% of the patients, no cause can be identified, and they are referred to as cases of an unexplained recurrent spontaneous abortion (URSA). However, it has been shown that URSA might be alloimmune in origin with failure of the fetal–maternal immunologic tolerance.[4-6]

Up to 15% of the patients with recurrent miscarriage have been found to be positive for antiphospholipid antibody

Access this article online

Quick Response Code:

Website: www.jhrsonline.org

DOI: 10.4103/jhrs.JHRS_65_16

Address for correspondence: Prof. Mostafa Fouad Gomaa, Abbasia Square Cairo Egypt, Ain Shams University Maternity Hospital First Floor, Cairo, Egypt.
E-mail: Mostafafouadg@gmail.com

How to cite this article: Gomaa MF, Elkhouly AG, Farghly MM, Farid LA, Awad NM. Uterine CD56^{dim} and CD16^{+} Cells in Refractory Antiphospholipid Antibody-Related Pregnancy Loss and Chromosomally Intact Abortuses: A Case–Control Study. J Hum Reprod Sci 2017;10:18-23.

syndrome (APS), and both low-dose aspirin and low molecular weight heparin have been recommended for the cases of obstetric APS.[7] Unfortunately, 30% of the cases continue to experience pregnancy loss in spite of treatment with no obvious cause and no effective treatment.[8]

Chromosomal aberrations in the embryo account only for 30% of the miscarriages in APS.[9,10] There is much evidence on the underlying inflammatory mechanisms, as the complement-mediated tissue injury and the infiltration of placental bed with high concentration of inflammatory cells suggest another mechanism for the pregnancy loss in pregnancies affected by APS.[11,12]

The pathophysiologic basis of obstetric APS is not fully understood. The pathological features of antiphospholipid antibodies (APAs) on the trophoblast include the following: decreased vasculosyncytial membranes, increased syncytial knots, fibrosis, and infarcts than in women without APS.[13] The changes in syncytial membranes may be secondary to thrombosis or secondary to placental damage by the antibodies themselves, which can inhibit placental human chorionic gonadotropin secretion,[14] cause complement activation,[15] and impair cytokine levels, but all of which may be responsible for fetal loss in obstetric APS. Cytokine imbalances are particularly relevant, as cytokines may affect the activation or inhibition of natural killer (NK) cells.[16]

The presence of APAs was shown to be associated with increased peripheral blood NK cells number, proportions, and cytotoxicity especially in a patient with RSA.[17]

APAs augment NK cell numbers and cytotoxicity, and result in an increased recruitment of decidual NK cells. Under these conditions, noncytotoxic decidual NK cells might change to cytotoxic $CD56^+/16^+$ NK cells, which in turn act via several mechanisms such as the mediation of trophoblastic tissue apoptosis and the secretion of various proinflammatory cytokines causing decidual microvessel thrombosis and fetal loss.[18]

This study aimed at evaluating the decidual NK cells in obstetric APS patients experiencing fetal loss in spite of low dose aspirin (LDA) and low molecular weight heparin (LMWH) treatment.

Patients and Methods

Selection of participants

The study was designed over a 4-year period from January 2012 to December 2015. One hundred eighteen cases having a history of obstetric APS and experiencing fetal loss in their current pregnancy in spite of low-dose aspirin (81 mg/day) and low molecular weight heparin (enoxaparin 40 mg/day) treatment were included in this study. The study was conducted at the recurrent miscarriage clinic of a university hospital. Another 32 patients undergoing elective termination of viable pregnancy before 20 weeks because of heart disease New York heart assiciation (NYHA) III and IV stages and due to lethal congenital anomalies as bilateral polycystic kidneys, renal agenesis, and anencephaly were taken as controls.

In our study, unexplained recurrent miscarriage was defined as ≥2 confirmed successive spontaneous miscarriages (<20 weeks' gestation). APS was diagnosed according to the Sydney criteria.[19] All the patients had their antibody status checked at the enrollment.

In our study, the women who proved to have septic miscarriage, documented with endocrinopathies (diabetes, thyroid disorders, and hyperprolactinemia), other autoimmune syndromes, mullerian anomalies, metabolic disorder, thrombophilia, abnormal karyotype in one or both parents, the history of hormonal contraception, and the history of intrauterine contraceptive device application within the last 3 months preceding current pregnancy were excluded from the study. The women more than 35 years old and those with body mass index (BMI) >30 were also excluded.

The women were included in the study after signing a written informed consent. The study was conducted in compliance with the declaration of Helsinki and approved by the hospital research ethical committee.

Technical information

For each patient, a thorough medical and obstetric history was taken, and physical examination was performed.

Evacuation and curettage were performed for all women included in this study; these two operations were performed under regional anesthesia, using suction evacuation to collect abortus specimens after cervical dilatation, followed by uterine wall curettage to collect decidua specimens. Abortus specimens were collected on a special medium for a long-term monolayer cell culture and subsequent chromosome analysis using a conventional Giemsa banding technique. Decidua specimens of cases with normal karyotyping of the abortus were subjected to an immunohistochemical staining using monoclonal antibodies specific to uterine natural killer (uNK) cells, namely $CD56^+$ and $CD16^+$.

Reagents and materials used included the following:

(1) Primary antibodies: monoclonal mouse antibody (MoAb) against $CD56^+$ and $CD16^+$ expressed on NK cells.

(2) Universal kits: immunodetection system from Biogenex Laboratories, Switzerland contained the following: (a) negative control antibody, (b) biotinylated antiimmunoglobulin for mouse antibody, (c) label: streptavidine peroxidase complex, (d) chromogen: 2,3-diaminobenzidine (DAB) chromogen solution, ready to use substrate buffer and H_2O_2 substrate for use with liquid DAB chromogen and substrate buffer, and (e) blocking reagent to block endogenous peroxidase activity.
(3) Lyophilized pepsin powder, phosphate buffer saline, counter stain (Mayer's hematoxylin), distilled water, and mounting media (Canada balsam).
(4) Staining jars, microscopic positively charged slides, cover slips for slides, and immune stainer.
(5) Light microscope: with 100× and 400× magnification.

Immunohistochemical procedure: Decidua specimens were fixed in 10% neutral buffered formalin for a period not more than 24 h, routinely processed and embedded in paraffin wax. Three-micrometer-thick sections were mounted onto 3-aminopropyltriethoxysilane (Sigma Chemical Co., Poole, UK) coated slides, and serial sections were stained for uNK cells ($CD56^+$ and $CD16^+$). All the primary antibodies were incubated for 60 min for $CD56^+$ and for 120 min for $CD16^+$ at room temperature, and the reaction was developed with 3,3-diaminobenzidine (2,3 diaminobenzidine (DAB); Sigma Chemical Co., Poole, UK) containing 0.01% H_2O_2 to give a brown reaction product; staining intensity was noted; sections were lightly counterstained with Mayer's hematoxylin, dehydrated, cleared, and mounted with DPX synthetic resin (Raymond A, Lamb Ltd., London, UK), and were then examined by using an ordinary light microscopy. Appropriate positive controls (neuroblastoma for $CD56^+$ and tonsils for $CD16^+$) were performed in each staining run, and negative controls were performed for each sample by replacing the primary antibody with mouse immunoglobulin-G.

Ethics

The study was conducted in accordance with the ethical standards of Helsinki declaration 1973 (revised 2000) and was approved by the local ethical committee of Ain Shams University Maternity Hospital.

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences version 18 software (SPSS Inc., Chicago, IL, United States). Numerical variables were presented as mean and standard deviation (±SD), whereas categorical variables were presented as number (n) and percentage (%). Chi-square and Fisher exact tests were used for making comparison regarding the qualitative variables between the groups. A difference with a P value <0.05 was considered statistically significant.

Required sample size was calculated using G*Power software version 3.17 for the sample size calculation (Heinrich Heine Universität, Düsseldorf, Germany), setting α-error probability at 0.05, power (1-β error probability) at 0.95%, and effective sample size (w) at 0.3. The effective size (w) was calculated as follows: $w = \sqrt{\chi^2/N}$, where χ^2 is the chi-square test, and N is the total sample size. The number of women participants needed to produce a statistically acceptable figure was 100.

Results

Among the one hundred eighteen women recruited for the study, 21 women were excluded. Among the excluded women, four women were excluded for having anatomical uterine defects, 10 women for having age >35 or BMI >30, four women for having history of chronic anovulation suggestive of polycystic ovarian disease, and three women with hyperprolactinemia and hypothyroidism. Abnormal fetal karyotype was found in nine (9/97) cases of the study group, which means that abnormal karyotype accounts for only 9.3% of the causes of failure of treatment. Abnormal karyotype was found in four cases of the control group. Only cases with the normal karyotype were subjected to the decidual uNK cells analysis. We found that $CD56^{dim}$ and $CD16^+$ cells were found in the decidua of 79 cases with chromosomally intact abortuses (79/88), which means that aberrant NK cells expression might account for 81.4% of the cases of refractory APA-mediated pregnancy loss.

The mean age of the women included in the analysis was 31.25 ± 2.09 years, and mean BMI was 27.34 ± 3.41 kg/m^2 [Table 1]. A karyotyping study of the abortus specimens showed normal karyotyping in 89.9% (116/129) of the studied specimens and abnormal karyotyping in 10.1% (13/129) of the studied specimens [Table 2]. From the 13 women with abnormal karyotype, nine were in the studied group (they are excluded) and four in the control group [Tables 3 and 4].

Table 1: Descriptive characteristics of the study group

Variables	Study group (n = 97)
Age	31.25 (20–35)
Previous miscarriage	4 (2–9)
Weight (kg)	89.6 (60–110)
BMI	27.34 (19–30)
GA at time of miscarriage (weeks)	10.6 (8–19)

GA, gestational age.

We found a significant difference between the expression of $CD56^{dim}$ and $CD16^{+}$ in the decidua of cases and controls with an odds ratio (OR) of 21.94, as shown in Table 5.

Discussion

With proper management, more than 70% of the patients with obstetric APS will have a live birth. The goals of treatment in obstetric APS are to improve maternal, fetal, and neonatal outcomes by decreasing the risks of the known complications of the disorder, including maternal thrombosis, fetal loss, preeclampsia, placental insufficiency, and fetal growth restriction.[20]

Table 2: Karyotyping analysis of the studied specimens

Variable	Number (*n*)	Percentage (%)
Normal female (46 xx)	96	74.4
Normal male (46 xy)	20	15.5
Abnormal karyotype	13	10.1
Triploidy	2	1.5
Tetraploidy	3	2.32
Aneuploidy	8	6.2
Total	129	100

Earliest treatment for recurrent pregnancy loss associated with obstetric APS was a combination of high-dose prednisone and low-dose aspirin, with successful outcome in 75% of the cases but with high maternal and fetal morbidity due to gestational diabetes, hypertension, and premature rupture of membranes. The combination of prednisone and aspirin was compared with the combination of heparin, and both were found to be equally efficacious with less morbidity in the heparin group.[21] However, up to 30% of women with an obstetric APS experience RSA in spite of the use of LDA and LMWH in treatment, and this failure might be due to the underlying inflammatory mechanisms including complement-mediated tissue injury and the higher concentration of various inflammatory cells in the deciduas of those patients.[8]

The most abundant immune cells in the uterine decidua around the time of implantation and early placental development are the uNK cells. Altered numbers of uNK cells have been associated with several human reproductive disorders, including recurrent miscarriage and recurrent implantation failure.[22]

This study was designed to evaluate the role of uNK cells in patients with refractory APA-mediated RSA. In the study, karyotyping results showed normal karyotyping in

Table 3: Descriptive characteristics of the study and control groups

Variables	Study group ($n = 97$)	Control group ($n = 32$)	*P* value
Age	28.7 ± 1.12 years	32.2 ± 2.2 years	Ns
Previous miscarriage	4 ± 1.4	1 ± 2.1	Hs
Weight	89.6 ± 7.2 kg	94.1 ± 6.5 kg	Ns
BMI	27.4 ± 3.1	30.4 ± 4.3	Ns
GA at time of miscarriage	10.6 ± 3.03 weeks	11.24 ± 2.54 weeks	Ns

GA, gestational age; Hs, highly significant; Ns, non significant.

Table 4: Characteristics of the study group according to the number of previous miscarriages

Variables	≤3 miscarriages (41 cases)	≥3 miscarriages (56 cases)	*P* value
Age	29.7 ± 2.12 years	30.2 ± 3.2 years	Ns
Weight	86.6 ± 9.2 kg	90.1 ± 4.6 kg	Ns
BMI	26.7 ± 6.2	32.6 ± 5.6	Ns
GA at time of miscarriage	9.6 ± 4.3 weeks	10.4 ± 2.03 weeks	Ns
Abnormal karyotype	6	7	Ns
$CD56^{dim}CD16^{+}$ expression	34	45	Ns

GA, gestational age; Ns, non significant.

Table 5: IHC results of decidua specimens in cases with chromosomally intact abortuses and controls

Variable	$CD56^{dim}CD16^{+}$ Number (%)	$CD56^{bright}CD16^{-}$ Number (%)	*P*-value	OR	95% confidence interval
Refractory APLAb$ (88 cases)	79 (89.77%)	9 (10.2%)	<0.0001	21.94	7.515–64.08
Control group (28 cases)	8 (28.6%)	20 (71.5%)			
Total	87	29			

IHC = immunohistochemical.

90.7% and abnormal karyotyping in 9.3% of the studied abortus specimens. A 29–57% rate of chromosomal abnormality was previously reported during analysis of miscarried tissue from the women suffering RSA,[9,23] and the higher percent of normal chromosomal results in miscarried tissue of women with RSA confirms that there may be other factors other than chromosomal abnormalities associated with RSA. However, the relatively lower percentage of chromosomal abnormalities in our study might be due to our inclusion criteria and the use of conventional G-banding technique in the analysis, which is less accurate than comparative genomic hybridization, and this might have caused some sort of bias in our analysis.

In our study, we found an overexpression of $CD56^{dim}$ and $CD16^{+}$uNK cells in the decidua specimens of women with refractory APA-mediated RSA. The findings of this study suggest that uNK cells might play an important role in the pathogenesis of refractory APA-mediated RSA. It is to be noted that Perricone *et al.*, in 2007, found that the peripheral NK cells were elevated in the patients with RSA and APS, and these cells might be the source of uterine natural killer cells. In their study, they hypothesized that there is a subcategory of APS that is associated with elevated NK cells, and this group is the one suffering from pregnancy losses during the first 10 weeks of gestation.[24] Our results confirm this hypothesis, as it proves the elevated levels of uNK cells in resistant APS raising the hypothesis that NK cells augment the effect of APAs on trophoblastic tissue and also raises a question on the benefit of steroids in the treatment of such patients. Prednisolone has been suggested as a suitable treatment for RSA women with high uNK cells,[25] and it has been tried with encouraging results in the cases of resistant obstetric APS.[8] The beneficial effects of steroids might be due to its suppressive effect on uNK cells.

Acknowledgements

The authors wish to thank all the women participated and included in this study, and the study was funded by the authors themselves.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Mei S, Tan J, Chen H, Chen Y, Zhang J. Changes of $CD4^{+}CD25^{high}$ regulatory T cells and FOXP3 expression in unexplained recurrent spontaneous abortion patients. Fertil Steril 2010;94:2244-7.
2. Beaman KD, Ntrivalas E, Mallers TM, Jaiswal MK, Kwak-Kim J, Gilman-Sachs A. Immune etiology of recurrent pregnancy loss and its diagnosis. Am J Reprod Immunol 2012;67:319-25.
3. Stephenson M, Kutteh W. Evaluation and management of recurrent early pregnancy loss. Clin Obstet Gynecol 2007;50:132-45.
4. Christiansen OB, Nielsen HS, Pedersen B. Active or passive immunization in unexplained recurrent miscarriage. J Reprod Immunol 2004;62:41-52.
5. Kwak-Kim J, Park JC, Ahn HK, Kim JW, Gilman-Sachs A. Immunological modes of pregnancy loss. Am J Reprod Immunol 2010;63:611-23.
6. Leber A, Zenclussen ML, Teles A, Brachwitz N, Casalis P, El-Mousleh T, *et al.* Pregnancy: Tolerance and suppression of immune responses. Methods Mol Biol 2011;677:397-417.
7. Bates SM, Greer IA, Pabinger I, Sofaer S, Hirsh J. Venous thromboembolism, thrombophilia, antithrombotic therapy, and pregnancy: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). Chest 2008;133:844-86S.
8. Bramaham K, Thomas M, Nelson-Piercy C, Khamashta M, Hunt BJ. First-trimester low-dose prednisolone in refractory antiphospholipid antibody-related pregnancy loss. Blood 2011;117:6948-51.
9. Ogasawara M, Aoki K, Okada S, Suzumori K. Embryonic karyotype of abortuses in relation to the number of previous miscarriages. Fertil Steril 2000;73:300-4.
10. Takakuwa K, Asano K, Arakawa M, Yasuda M, Hasegawa I, Tanaka K. Chromosome analysis of aborted conceptuses of recurrent aborters positive for anticardiolipin antibody. Fertil Steril 1997;68:54-8.
11. van Horn JT, Craven C, Ward K, Branch DW, Silver RM. Histologic features of placentas and abortion specimens from women with antiphospholipid and antiphospholipis-like syndromes. Placenta 2004;25:642-8.
12. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, *et al.* International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost 2006;4:295-306.
13. Out HJ, Kooijman CD, Bruinse HW, Derksen RH. Histopathological findings from patients with intrauterine fetal death and antiphospholipid antibodies. Eur J Obstet Gynecol 1991;41:179-86.
14. Shurtz-Swirsky R, Inbar O, Blank M, Cohen J, Bakimer R, Barnea ER, *et al. In vitro* effect of anticardiolipin autoantibodies upon total and pulsatile placental hCG secretion during early pregnancy. Am J Reprod Immunol 1993;29:206-10.
15. Salmon JE, Girardi G. The role of complement in the antiphospholipid syndrome. Curr Dir Autoimmun 2004;7:133-48.
16. Fishman P, Falach-Vaknine E, Zigelman R, Bakimer R, Sredni B, Djaldetti M, *et al.* Prevention of fetal loss in experimental antiphospholipid syndrome by *in vivo* administration of recombinant interleukin-3. J Clin Invest 1993;91:1834-7.
17. Lash GE, Bulmer JN. Do uterine natural killer (uNK) cells contribute to female reproductive disorders? J Reprod Immunol 2011;88:156-64.
18. Quenby S, Farquharson R. Uterine natural killer cells, implantation failure and recurrent miscarriage. Reprod Biomed Online 2006;13:24-8.

19. The American College of Obstetricians and Gynecologists. Antiphospholipid syndrome. ACOG Pract Bull 2011;118:1-8.
20. Branch DW, Khamashta MA. Antiphospholipid syndrome: Obstetric diagnosis, management, and controversies. Obstet Gynecol 2003;101:1333-44.
21. Cowchock FS, Reece EA, Balaban D, Branch DW, Plouffe L. Repeated fetal losses associated with antiphospholipid antibodies: A collaborative randomized trial comparing prednisone with low dose heparin treatment. Am J Obstet Gynecol 1992;166:1318-27.
22. Seshadri S, Sunkara SK. Natural killer cells in female infertility and recurrent miscarriage: A systematic review and meta-analysis. Hum Reprod Update 2014;20:429-38.
23. Carp H, Toddler V, Aviram A, Daniely M, Mashiach S, Barkai G. Karyotype of the abortus in recurrent miscarriage. Fertil Steril 2001;75:678-82.
24. Perricone C, De Carolis C, Giacomelli R, Zaccari G, Cipriani P, Bizzi E, *et al.* High levels of NK cells in the peripheral blood of patients affected with anti-phospholipid syndrome and recurrent spontaneous abortion: A potential new hypothesis. Rheumatology (Oxford) 2007;46:1574-8. Epub 2007 Aug 17.
25. Quenby S, Kalumbi C, Bates M, Farquharson R, Vince G. Prednisolone reduces preconceptual endometrial natural killer cells in women with recurrent miscarriage. Fertil Steril 2005;84:980-4.