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Original Article

Complement activation fragment C3a is a sensitive biomarker for patients with recurrent miscarriage

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Abstract: Purpose: This study aimed to investigate complement activation in recurrent miscarriage (RM) and to identify biomarkers for RM. Methods: A total of 108 female patients who were diagnosed with RM and 120 control female volunteers for reproductive health screening were included in this study. ELISA was used to measure plasma levels of the complement activation fragments C3a, C4a, C5a, and serum levels of anticardiolipin antibodies and anti-β2 glycoprotein I. Nephelometric immunoassays were used to measure serum C3 and C4 levels. Results: All complement activation fragment (C3a, C4a and C5a) levels were significantly higher in RM patients than in controls ($P < 0.05$). Only C3a levels were significantly higher in RM patients with normal C3 or C4 levels than in controls ($P < 0.05$). C3a levels were significantly higher in RM patients with or without antiphospholipid antibodies than in controls ($P < 0.05$). Conclusion: Complement activation is a common event that occurs in RM patients. Additionally, the complement activation fragment C3a is a sensitive biomarker for RM patients.

Keywords: Recurrent miscarriage, antiphospholipid syndrome, antiphospholipid antibodies, complements activation, C3a

Introduction

Complement is one of the first lines of defense in innate immunity and is important for cellular integrity, tissue homeostasis, and modifying the adaptive immune response [1]. Complement-mediated innate immune function not only recognizes and eliminates infectious agents, but also controls homeostatic processes, such as clearance of cellular debris and apoptotic cells. Generally, there are three pathways for complement activation: the classical pathway, the lectin pathway, and the alternative pathway. The classical pathway is primarily activated by antigen-antibody complexes, and the other two pathways are activated by carbohydrate groups and bacterial surfaces [2]. Activation of the complement cascade leads to opsonization of the target by C3b/C4b/C5b, followed by generation of the lytic membrane attack complex, which releases pro-inflammatory anaphylatoxins (C3a, C4a, and C5a) in blood to attract leukocytes. Therefore, the complement activation fragments C3a, C4a, and C5a in blood may reflect the status of complement activation in the whole body.

Recently, in recurrent miscarriage (RM) patients with antiphospholipid syndrome (APS) had been found to be implicated with complement activation [3-6], Complement activation is required for antiphospholipid antibodies (APLS)-induced fetal loss [3, 5, 7]. Furthermore, heparin prevents APLS-induced fetal loss by inhibiting complement activation [4, 6]. These studies suggest that complement activation is a critical event for APLS-induced fetal loss or RM.

RM is traditionally defined as three or more consecutive miscarriages occurring prior to 20 weeks of post-menstruation [8-11]. No studies have determined if complement activation is also involved in RM patients without APS. Furthermore, some studies have suggested that levels of C3, C4, or C3a may predict pregnant results for RM patients with or without APS [4, 12]. Therefore which complement or complement activation component (including C3, C4, and complement activation fragments C3a, C4a, and C5a) is a sensitive tool for evaluating complement activation needs to be determined.

Table 1. Baseline characteristics of controls and recurrent miscarriage (RM) patients

	RM (n = 108)	Healthy control (n = 120)	P-value
Age (years)	30.04±4.87	29.85±4.17	0.854
Number of previous abortions	3.53±0.77	None	0.000
Body mix index	22.85±2.22	22.40±2.57	0.418
Age of marriage	26.36±3.84	25.13±2.95	0.104
Estradiol (pmol /L)	193±74	174±97	0.292
Progesterone (nmol/L)	2.28±1.09	2.15±0.81	0.151
Cigarette smoking	None	None	-
Alcohol consumption	None	None	-
Exposed to any harmful substance	None	None	-

The independent samples t-test was used for analysis.

Materials and methods

Patients

This prospective study was performed during July 2009 to December 2013. A total of 108 female patients (range, 20-36 years old) who were diagnosed with RM were included. We also included 120 control female volunteers for reproductive health screening who were recruited from the outpatient clinic of the Department of Reproductive Medicine at the First Affiliated Hospital of Xi'an Jiaotong University Medical College.

RM was diagnosed according to the history of patients who had experienced three or more consecutive spontaneous miscarriages within 12 weeks of gestation [13]. All of the patients received standard evaluations consisting of thrombophilia screening, parental karyotypic evaluation, endocrine screening, and an ultrasonographic examination for possible uterine anomalies. Women with any thrombophilic, endocrine, karyotypic, or anatomical abnormalities were excluded from the patient group. All healthy female volunteers with at least one live birth and no history of pregnancy loss were enrolled as controls from the population who came for healthy screening. There was no significant difference in age between the patient and control groups (**Table 1**). None of the participants had complications associated with infection, malignancy, impaired circulation or tissue ischemia, a history of cigarette smoking and alcohol consumption, or exposure to any harmful substance in the recent 2 months prior to the date of blood collection. The study was conducted in accordance with the Declaration of Helsinki and the Principles of Good Clinical Practice. The study procedures were approved by the institutional review board and ethics committee, and written informed consent was obtained from all participants.

Collection of serum and plasma samples

Venous blood of RM patients and controls after overnight fasting (approximately 10-12 h) was obtained for serum and plasma collection on the 3rd day of the menstrual cycle to minimize hormonal effects, and after 3 months of their last miscarriage or delivery. For serum samples, venous blood was collected after 1500× g centrifugation for 10 min at room temperature, and samples were stored at -80°C until later analysis. For plasma samples, venous blood was collected in 3.8% sodium citrate (9:1) and centrifuged twice at 2000× g for 15 min at 4°C. Obtained plasma was stored at -80°C until later analysis. Repeated freezing and thawing of specimens was avoided, and plasma was stored before performing enzyme-linked immunoassay (ELISA) for complement activation within 1 year.

Detection of C3a, C4a, C5a, C3, and C4 levels

Plasma C3a, C4a, and C5a levels were measured with an ELISA using monoclonal antibodies specific to human C3a-desArg, C4a-desArg, and C5a-desArg, with low detection limits of 0.007 ng/ml, 0.006 ng/ml, and 0.047 ng/ml, respectively. Immunoassay systems for C3a (human C3a BD OptEIA™ ELISA), C4a (human C4a BD OptEIA™ ELISA), and C5a (human C5a BD OptEIA™ ELISA II) were obtained from BD Biosciences (San Jose, CA, USA). Complement C3a, C4a, and C5a assays were performed with 1000× diluted plasma according to the manufacturer's instructions. The plates were read at 450 nm by a Bio-Rad microplate reader with the wavelength correction set at 570 nm (iMark™ Microplate Absorbance Reader; Bio-Rad Laboratories, Hercules, CA, USA). The amount of C3a, C4a, and C5a in each plasma sample was quantified by interpolation

Table 2. Positive rates of antiphospholipid antibodies (APLS), Fibrin(ogen) degradation product (FDP) and D-dimer levels in controls and recurrent miscarriage (RM) patients

	RM (n = 108)	Healthy control (n = 120)	P-value
APLS	26.9% (29/108)	9.2% (11/120)	0.000
FDP	5.6% (6/108)	4.2% (5/120)	0.625
D-dimer	8.3% (9/108)	10.0% (12/120)	0.664

The chi-squared test was used for analysis.

from individual standard curves composed of purified human C3a, C4a, or C5a. The calculated inter- and intra-assay coefficients of variation were all less than 10%. Serum C3 and C4 levels were determined with nephelometric immunoassays using goat anti-human C3 and goat anti-human C4 (Maike BioTeck, Sichuan, China), with normal ranges of 0.8-1.8 g/L and 0.2-0.4 g/L, respectively.

Anti-phospholipid antibody testing

Lupus anticoagulant (LA), anticardiolipin antibodies (ACAs, IgG and IgM), and anti-β2 glycoprotein I (anti-β2 GPI, IgG and IgM) were recognized as APS-related autoantibodies in the revised international consensus statement for definite APS. Serum levels of ACAs (IgG and IgM) and anti-β2 GPI (IgG and IgM) were detected with an ELISA test kit (Euroimmun Medizinische Labordiagnostika AG, Lubeck, Germany). All of the procedures were performed according to the manufacturer's instructions. The plates were read at 450 nm by a Bio-Rad microplate reader (Bio-Rad). The levels of serum ACAs and anti-β2-GPI were quantified with standard curves. The normal range of ACAs IgG and IgM is below 12 RU/ml (positive sample ≥ 12 RU/ml), and the normal range of anti-β2-GPI IgG and IgM is below 20 RU/ml (positive sample ≥ 20 RU/L). LA was detected using a panel of two tests, the simplified dilute Russell's viper venom time test and an LA-sensitive test for activated partial thromboplastin time (APTT). The simplified dilute Russell's viper venom time test was carried out with a kit from Siemens (Siemens, Germany) using the CA-1500 coagulation Analyzer (Sysmex Corporation, Japan). The LA-sensitive test for APTT was performed with a kit from Sysmex Corporation. LA was finally determined to be present or absent based on mixing studies and phospholipid dependence. ACAs (IgG and IgM), anti-β2 GPI (IgG and IgM), and LA needed to be present in a high titer on two or more occasions at least 12 weeks apart.

Detection of D-dimer and fibrin (ogen) degradation product

A hypercoagulation state or thrombus formation is recognized as a common event in RM. Fibrin (ogen) degradation product (FDP) and D-dimer are two classic markers of a hypercoagulation state. After collection of plasma, D-dimer and FDP levels were immediately determined. D-dimer and FDP levels were measured by the Bead solidification method using a blood coagulation analyzer (C2000-4; Stago, France). The normal range of D-dimer levels is 0-1.0 mg/L (positive sample > 1.00 mg/L) and the normal range of FDP levels is 0-5 mg/L (positive sample > 5.00 mg/L). All of the procedures were performed according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using SPSS software version 13.0 (SPSS Inc., Chicago, IL). The unpaired Student's *t*-test, ANOVA, and chi-squared tests were used as appropriate. Continuous variables are shown as the mean ± SD. $P < 0.05$ was defined as statistically significant.

Results

APLS, D-dimer, and FDP levels in RM patients and controls

LA/anti-β2-GPI and/or ACAs (IgG or IgM) were found more frequently in RM patients (26.9%, 29/108) compared with controls (9.2%, 11/120, $P < 0.001$, **Table 2**). This finding suggested that APLS were a trigger for a small part of RM. Only 26.9% RM patients suffered from APS in our study. Furthermore, there were no significant differences in the levels of D-dimer ($P = 0.625$) and FDP ($P = 0.664$) between RM patients and controls, suggesting that D-dimer and FDP could not be used as a monitoring tool for RM.

Complements and their activation fragments in RM patients

There was no significant difference in C4 levels between RM patients (0.24±0.09 g/L) and con-

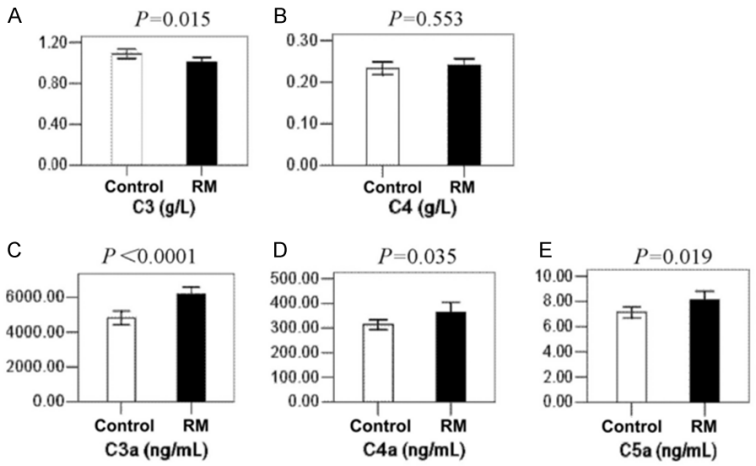

Figure 1. Serum levels of complement C3, C4, C3a, C4a and C5a in recurrent miscarriage patients and controls. The levels of C3 (A), C4 (B), C3a (C), C4a (D) and C5a (E) in recurrent miscarriage patients and controls are shown as mean ± SD. The *P*-values are marked on the top of each bar. RM, recurrent miscarriage.

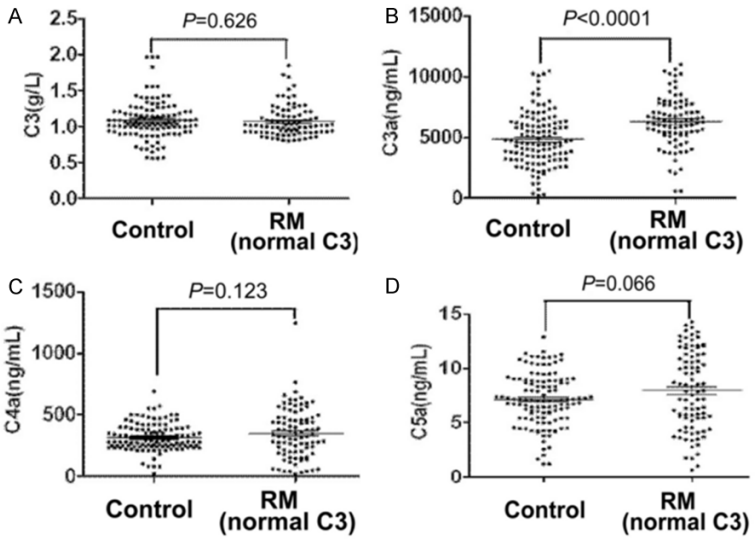

Figure 2. The comparison of C3, C3a, C4a and C5a between the healthy controls and recurrent miscarriage patients with normal C3 level. Comparisons of C3 (A), C3a (B), C4a (C) and C5a (D) levels between healthy controls and recurrent miscarriage patients with normal C3 level. *P*-values are marked on the top of the bar. RM, recurrent miscarriage.

trols (0.23±0.08 g/L, $P = 0.553$). However, C3 levels were significantly lower in RM patients (1.01±0.24 g/L) than in controls (1.09±0.26 g/L, $P = 0.015$). These findings suggested that C3, but not C4, was a sensitive indicator of immune reactions in RM patients. Moreover, C3a (6189±2126 ng/ml vs. 4815±2177 ng/ml, $P < 0.001$), C4a (362±214 ng/ml vs. 314±109 ng/ml, $P = 0.035$), and C5a (8.12±3.62 ng/ml vs. 7.15±2.42 ng/ml, $P = 0.019$) were significantly elevated in RM patients compared with controls (**Figure 1** and Supplementary Table 1). Taken together, these findings suggested that complement activation fragments (C3a, C4a, and C5a), but not complements (C3 and C4), were more sensitive for measuring complement activation in RM, although, C3 levels, but not C4 levels, were significantly different between RM patients and controls.

C3a is a sensitive biomarker for RM patients with normal C3/C4 levels

To determine whether C3a, C4a, and C5a are sensitive enough to distinguish RM patients from controls with normal C3 levels, plasma levels of C3a, C4a, and C5a were compared between 120 controls and 89 RM patients with normal C3 levels (**Figure 2** and Supplementary Table 2). There were no significant differences in C3, C4a, and C5a levels between the two groups ($P > 0.05$). However, C3a levels were significantly higher in RM patients with normal C3 levels (6289±2084 ng/ml) than in controls (4815±2177 ng/ml, $P < 0.001$). This finding suggested that the complement activation fragment C3a was a sensitive candidate biomarker for RM patients with normal C3 levels.

C3, C3a, C4a and C5a levels were compared between 120 controls and 72 RM patients with normal C4 levels (**Figure 3** and Supplementary Table 3). There were no significant differences in C3, C4a, and C5a levels between the two groups. Only C3a was sensitive enough to distinguish RM patients (6285±2047 ng/ml) with normal C4 levels from controls (4815±2177 ng/ml, $P < 0.001$). This finding indicated that

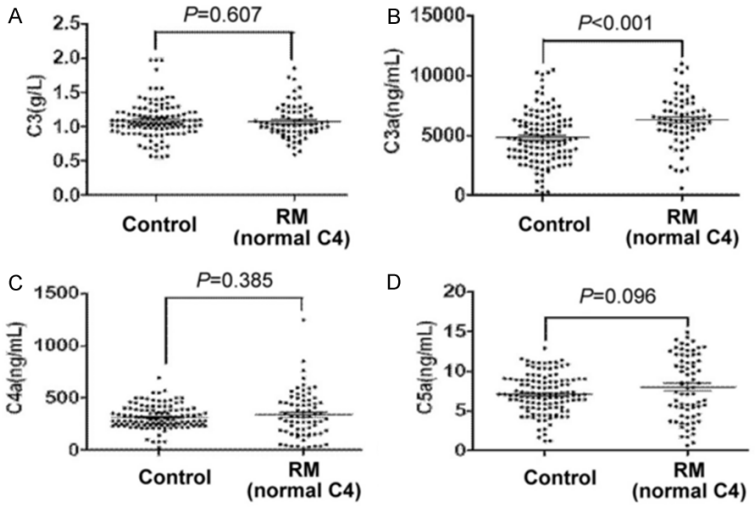

Figure 3. The comparison of C3, C3a, C4a and C5a between the healthy controls and recurrent miscarriage patients with normal C4 level. Comparisons of C3 (A), C3a (B), C4a (C) and C5a (D) levels between healthy controls and recurrent miscarriage patients with normal C4 level. *P*-values are marked on the top of each bar. RM, recurrent miscarriage.

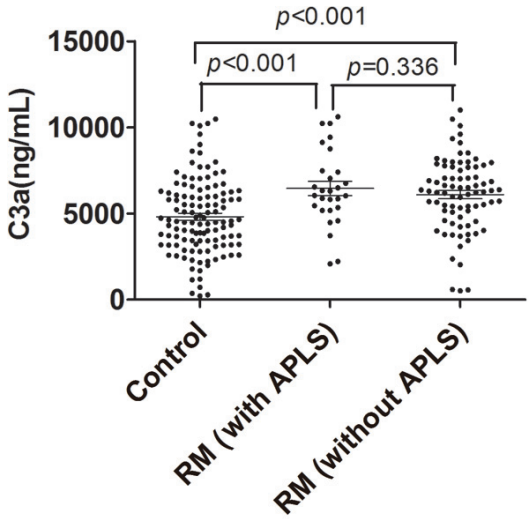

Figure 4. The comparison of C3a among healthy controls, recurrent miscarriage patients with or without APLS. The level of C3a in the healthy controls and recurrent miscarriage patients with or without APLS are shown as above. *P*-values are marked on the top of each bar. RM, recurrent miscarriage.

C3a was a sensitive biomarker for RM patients with normal C4 levels.

C3a is a sensitive biomarker not only for RM patients with APS, but also for RM patients without APS

RM patients with any one of the APLS (LA, anti-β2-GP-1 IgG or IgM, ACAs IgG or IgM) may be diagnosed as having APS. In the present study, C3a levels were significantly elevated in 29 RM patients with APLS (6499±2156 ng/ml) compared with 120 controls (4815±2177 ng/ml, $P < 0.001$), furthermore, C3a levels were significantly higher in 79 RM patients without APLS (6075±2118 ng/ml) than in the 120 controls ($P < 0.001$). There was no difference in C3a levels between RM patients with and without APLS ($P = 0.366$) (**Figure 4** and Supplementary Table 4). This result is in accordance with a previous study, which showed that complement was activated in RM patients with primary APS (15). These findings indicated that complement activation was a common biomarker of RM patients, regardless of whether they had APLS.

Discussion

RM affects up to 1-5% of all pregnant couples and may be induced by multiple etiologies. To identify some common characteristics in most RM patients, RM patients with anatomical uterine abnormalities, endocrine disorders, as well as chromosomal disorders in any of the parents, were excluded from this study. Therefore, in RM patients who were recruited in this study, the cause of RM was mainly attributed to APLS and unexplained etiology. Actually, only 29 of 108 (26.9%) RM patients in this study suffered from APS according to recommended APS guidelines by Jauniaux et al [14]. Complement activation fragment C3a levels were much more elevated in RM patients with APS than those in controls, which is consistent with many previous studies [3]. Complement activation is required in APS-induced fetal loss [3, 5, 15], and inhibiting complement activation through heparin treatment prevents APS-related pregnancy loss [6]. These findings suggest that complement activation is a critical pathophysiological process in APLS-induced fetal loss. Our results indicated that complement activation is a common event in RM patients with APS. Furthermore, for the first time, our

study showed that complement activation fragment C3a levels were more highly elevated in RM patients without APS than those in controls. This finding indicated that complement activation is an event that not only occurs in RM patients with APS, but also occurs in patients without APS. Therefore, our study suggests that complement activation may be an important process in all cases of RM.

Complement components and their activated fragments have been evaluated as diagnostic and predictive markers for several diseases and outcomes of pregnancy [4, 12, 16-19]. In our study, we systemically compared C3 and C4, and the complement activation fragments C3a, C4a, and C5a in RM patients and controls. We found that C3a could distinguish all RM patients from controls, regardless of whether RM patients suffered from APS, or whether they had low or normal C3 or C4 levels. Therefore, the complement fragment C3a is the most sensitive marker to evaluate complement activation in RM patients.

A hypercoagulation state or thrombus formation has been recognized as a common event in RM. However, FDP and D-dimer, two classic markers of the hypercoagulation state, were not different between RM patients and controls in our study. This finding suggests that these markers cannot be used to monitor RM. C3a and C5a are two necessary complement activation components that are released from all complement activation pathways. Because C5a is rapidly degraded and has a short half-life *in vitro*, C5a is difficult to use as a monitoring tool. For the first time, our study indicated that C3a was the most sensitive indicator to evaluate complement activation in patients with RM. More studies are required in the future to determine if C3a is an ordinary and sensitive indicator that can used for monitoring development and progression of RM patients with and without APS.

In conclusion, our study shows that activation of complements is universally elevated in patients with RM. Additionally, the complement activation fragment C3a is an effective and sensitive biomarker in RM patients. This finding provides a novel possibility to explore complement C3a as a clinical tool to evaluate the development and progression of RM.

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Disclosure of conflict of interest

None.

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Supplementary Table 1. C3, C4, C3a, C4a, and C5a levels in controls and recurrent miscarriage (RM) patients

	RM (n = 108)	Healthy control (n = 120)	P-value
C3 (g/L)	1.01±0.24	1.09±0.26	0.015
C4 (g/L)	0.24±0.09	0.23±0.08	0.553
C3a (ng/ml)	6189±2126	4815±2177	0.000
C4a (ng/ml)	362±214	314±109	0.035
C5a (ng/ml)	8.12±3.62	7.15±2.42	0.019

The independent samples t-test was used for analysis.

Supplementary Table 2. C3, C3a, C4a, and C5a levels in controls and recurrent miscarriage (RM) patients with normal C3 levels

	RM with normal C3 (n = 89)	Healthy control (n = 120)	P-value
C3 (g/L)	1.07±0.22	1.09±0.26	0.626
C3a (ng/ml)	6289±2084	4815±2177	0.000
C4a (ng/ml)	350±205	314±109	0.132
C5a (ng/ml)	7.96±3.59	7.15±2.42	0.066

The independent samples t-test was used for analysis.

Supplementary Table 3. C3, C3a, C4a, and C5a levels in controls and recurrent miscarriage (RM) patients with normal C4 levels

	RM with normal C4 (n = 72)	Healthy control (n = 120)	P-value
C3 (g/L)	1.07±0.25	1.09±0.26	0.607
C3a (ng/ml)	6285±2047	4815±2177	0.000
C4a (ng/ml)	338±220	314±109	0.385
C5a (ng/ml)	8.00±3.90	7.15±2.42	0.096

The independent samples t-test was used for analysis.

Supplementary Table 4. C3a levels in controls and in recurrent miscarriage (RM) patients with or without antiphospholipid antibodies (APLS)

	RM with APLS (n = 29)	RM without APLS (n = 79)	Healthy control (n = 120)
C3a (ng/ml)	6499±2156	6075±2118	4815±2177

One way ANOVA was used for analysis. RM patients with APLS versus controls: $P < 0.001$. RM patients without APLS versus controls: $P < 0.001$. RM patients with APLS versus RM patients without APLS: $P = 0.336$.