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Original articles – Obstetrics

Unexplained intrauterine fetal death is accompanied by activation of complement

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Abstract

Objective: Activation of the complement system has recently been implicated in the mechanisms of fetal loss in the antiphospholipid syndrome. It is, however, possible that complement activation is also involved in other causes of fetal death in the second and third trimesters of pregnancy. We therefore conducted a study to determine whether fetal death is associated with changes in the maternal plasma concentrations of complement split products or anaphylatoxins (C3a, C4a and C5a).

Study design: A cross-sectional study was designed to include normal pregnant women (n = 60) and patients with fetal death (n = 60). Patients with fetal death were classified according to the cause of fetal demise into: a) unexplained (n = 44); b) associated with preeclampsia (n = 8); and c) associated with chromosomal abnormalities or major congenital fetal anomalies (n = 8). The plasma concentrations of C3a, C4a and C5a were measured using sensitive and specific ELISAs. Non-parametric statistics were used for analysis. A P value of < 0.05 was considered significant.

Results: 1) The median plasma concentration of C5a was higher in patients with fetal death than in normal pregnant women [median 16 ng/mL (range 4.5–402.5) vs. median 11.6 ng/mL (range 1.2–87.1), respectively; $P < 0.001$]; 2) patients with an unexplained fetal death and those associated with preeclampsia had a higher median plasma C5a concentration than normal pregnant women ($P = 0.002$ and $P < 0.001$, respectively); 3) no differences were observed in the maternal plasma concentrations of C3a and C4a among the study groups.

Conclusions: Unexplained fetal death is associated with evidence of complement activation.

Keywords: Pregnancy; intrauterine fetal death; anaphylatoxins; complement system; C3a; C4a; C5a; preeclampsia.

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Introduction

The innate arm of the immune system, which is composed of humoral and cellular elements, represents the first line of defense against pathogens, non-self antigens or "danger signals" [23, 52]. The complement system plays a central role in innate immunity by inducing chemotaxis of inflammatory cells, clearance of immune complexes, opsonization, and direct lysis of microorganisms and/or other foreign cells [54, 90]. Moreover, a role for complement in orchestrating the adaptive immune response has been proposed [7].

The complement system can be activated by the classical, alternative or lectin pathways [89, 90]. The cleavage of C3, C4 and C5 results in the release of small peptides known as anaphylatoxins, which include C3a, C4a and C5a [18]. These anaphylatoxins can induce vascular permeability [8, 31], chemotaxis of inflammatory cells [11, 36, 74], and smooth muscle contraction [8, 14, 31]. In addition, phagocytic cells can generate C5a through the actions of proteolytic enzymes [43, 86, 87, 92]. Several studies have demonstrated that excessive or unregulated production of complement anaphylatoxins can contribute to tissue damage in conditions such as ischemia/reperfusion injury [97], sepsis [10, 91], rheumatoid arthritis [32], and adult respiratory distress syndrome [77].

Pregnancy is characterized by the expression of paternal alloantigens in fetal tissue that can elicit both a humoral [12, 13] and a cellular immune response [101].

Yet most pregnancies are successful, suggesting that there is tolerance to these alloantigens [78]. The specific mechanisms responsible for tolerance are not fully understood. However, a role for regulatory T-cells [4], complement regulatory proteins [99], and indolamine 2,3 dioxygenase (IDO) [55] has been proposed. Some pregnancy losses have been attributed to a dysregulated maternal immune response against fetal antigens [37]. Specifically, complement activation has been implicated in embryonic death [27, 50, 99]. Xu et al. [99] demonstrated that the deficiency of Crry, a membrane-bound complement regulatory protein expressed in mouse trophoblast, results in embryonic death with deposition of complement proteins and inflammatory cell infiltration within the placenta. Furthermore, Girardi et al. [25] demonstrated that knock-out mice for C5 and neutralization of C5a with antibodies against this anaphylatoxin or its receptor could prevent fetal damage and pregnancy loss in an animal model of antiphospholipid syndrome (APS). Therefore, activation of complement may be involved in the mechanisms responsible for fetal death. This study was conducted to determine whether the concentrations of anaphylatoxins in maternal blood change in patients with a fetal death.

Patients and methods

Study design

A cross-sectional study was conducted by searching our clinical database and bank of biological samples. This study was designed to determine the plasma concentration of C3a, C4a and C5a in normal pregnant women (n = 60) and pregnant women with a diagnosed fetal death (n = 60). Inclusion criteria for normal pregnancy included: 1) absence of medical, obstetrical or surgical complications; 2) absence of labor; 3) gestational age ranging from 20 to 41 weeks; and 4) delivery of a term infant, appropriate for gestational age. Women in this group were enrolled from either a labor-delivery unit (in cases of scheduled cesarean section) or our antenatal clinic. Fetal death was defined as the death of the fetus after the 20th week of gestation, confirmed by ultrasound examination. This group was sub-classified according to the causes of fetal death into: a) fetal death associated with known chromosomal abnormalities or major fetal anomalies (n = 8); b) fetal death associated with preeclampsia (n = 8); and c) unexplained fetal death (n = 44). Fetal chromosomal and structural anomalies in the first group included trisomy 21 (n = 3), trisomy 13 (n = 1), non-immune hydrops fetalis (n = 3), and ventricular septal defect with single umbilical artery (n = 1). Preeclampsia was defined as hypertension (systolic blood pressure of $\geq$140 mm Hg or diastolic blood pressure of $\geq$90 mm Hg on at least two occasions, 4 h to one week apart) and proteinuria ($\geq$ 300 mg in a 24 h urine collection or one dipstick measurement of $\geq$ 2+). Unexplained fetal death was defined as the absence of preeclampsia, chromosomal abnormalities, and major congenital anomalies, as documented in the autopsy report. Lupus anticoagulant and anticardiolipin antibody titers were determined. APS was defined using the international classification criteria [95, 96]. Transabdominal amniocentesis for karyotype determination was offered in view of the evidence that the likelihood of a successful karyotype determination is higher by studying amniotic fluid cells than fetal tissues in fetal death [6, 41, 67]. Amniotic fluid samples were sent for aerobic, anaerobic and Mycoplasma species cultures. All women provided written informed consent prior to the collection of samples. The collection of samples was approved by the IRBs of both Wayne State University and the National Institute of Child Health and Human Development (NIH). Many of these samples have been used in previous studies.

Blood collection

Samples of peripheral blood were collected in tubes containing EDTA (ethylene diamine tetraacetic acid). The samples were centrifuged and stored at -70°C. Specific and sensitive enzyme-linked immunoassays (ELISAs) were used to determine the concentrations of complement C3a, C4a and C5a. Immunoassay systems for C3a and C4a were obtained from Assay Designs, Inc. (Ann Arbor, MI). C5a immunoassays were obtained from American Laboratory Products Company (Windham, NH). Complement C3a, C4a and C5a assays were performed following the manufacturers' recommendations. Briefly, maternal plasma samples were incubated in duplicate wells of microtiter plates, which had been pre-coated with antigen specific (C3a, C4a or C5a) antibodies. C3a, C4a or C5a present in the standards or maternal plasma samples were immobilized by their specific pre-coated antibodies (forming antigen antibody complexes) during this incubation. Repeated washing and aspiration was conducted to remove unbound materials from the assay plates. This step was followed by incubation with a specific antibody-enzyme reagent. Following a wash step to remove excess unbound materials, a substrate solution was added to the wells of the microtiter plates, and color developed in proportion to the amount of antigen bound in the initial step of the individual assay. The color development was stopped with the addition of an acid solution, and the intensity of color was read using a programmable microtiter plate spectrophotometer (Ceres 900 Microplate Workstation, Bio-Tek Instruments, Winooski, VT). The concentrations of complement C3a, C4a or C5a in maternal plasma samples were determined by interpolation from individual standard curves composed of purified human C3a, C4a or C5a. The calculated inter-assay coefficients of variation (CV) for C3a, C4a and C5a immunoassays in our laboratory were 5.4%, 6.1% and 4.0%, respectively. Calculated intra-assay CV for C3a, C4a and C5a were 6.6%, 6.9% and 2.3%, respectively. The detection limit (sensitivity) was 0.13 ng/mL for C3a, 0.32 ng/mL for C4a, and 0.06 ng/mL for C5a.

Statistical analysis

Shapiro-Wilk tests were used to determine whether the data were normally distributed. A Kruskal-Wallis test was utilized for comparisons among study groups. Mann-Whitney U tests were used for post-hoc comparisons using the Bonferonni correction. Chi-square was used to compare proportions. The statistical package employed was SPSS 12 (SPSS Inc., Chicago, IL). A P value of <0.05 was considered statistically significant.

Table 1 Clinical characteristics of women with normal pregnancy and women with fetal death.

	Normal pregnancy n=60	IUFD n=60	P
Maternal age (y)	24 (17-37)	25.5 (17-41)	0.3
Nulliparity	18 (30)	24 (40)	0.2
Smoking	9 (15)	18 (30)	0.04*
Gestational age at venipuncture (wks)	31.1 (20.0-40.5)	30.9 (20.1-40.5)	0.7
Gestational age at delivery (wks)	39.5 (37-42)	31 (20.5-40.7)	<0.001*
Birthweight (g)	3320 (2610-3990)	1390 (140-5755)	<0.001*

IUFD, intrauterine fetal death.
Values are expressed as median (range) or number (percent).
* Statistically significant, $P<0.05$.

Results

Table 1 describes the clinical and obstetrical characteristics of the women in each group. Smoking was more frequent among patients with intrauterine fetal death than among those with uncomplicated pregnancies (30% vs. 15%; $P=0.04$). The median gestational age at delivery and birthweight were higher in the control group than in the fetal death group ($P<0.001$).

Overall, the median plasma concentration of C5a in patients with fetal death was higher than that of normal pregnant women ($P<0.001$; Figure 1C). No significant differences were observed in the median plasma concentrations of C3a and C4a between the two groups ($P=0.1$ and $P=0.2$, respectively; Figures 1A and 1B).

Patients with unexplained fetal death and those with fetal death associated with preeclampsia had a higher median plasma C5a concentration than normal pregnant women ($P=0.002$ and $P<0.001$, respectively; Figure 2C). However, women with fetal death associated with preeclampsia had a higher median plasma C5a concentration than those with unexplained fetal death ($P=0.003$; Figure 2C). There was no significant difference between the median plasma C5a concentrations of women with fetal death associated with a fetal anomaly and those of the other study groups (Figure 2C). Though not a frequent cause of fetal death, a case with ventricular septal defect and single umbilical artery was included in the fetal congenital anomaly group. However, the exclusion of this case from the analysis did not affect the results (data not shown). The median plasma concentrations of C3a and C4a were not different among the study groups (Figures 2A and 2B).

Amniotic fluid samples were obtained in 66% (29/44) of patients with unexplained fetal death. Microbial cultures of amniotic fluid samples from patients with unexplained fetal death were negative for aerobic and anaerobic bacteria, as well as Mycoplasma species.

Antiphospholipid antibody tests (lupus anticoagulant and/or anticardiolipin antibody titers) were determined in 42/44 patients with unexplained fetal death. Two patients from the unexplained fetal death group had APS. The results did not change after exclusion of these patients from the analysis (data not shown).

Discussion

Principal findings of the study

This study demonstrates that pregnant women with unexplained fetal death have a higher median plasma C5a concentration, but not of C3a or C4a, than normal pregnant women. These results are novel, as maternal plasma concentrations of anaphylatoxins have not previously been studied in association with human fetal death.

Activation and biological effects of the anaphylatoxins

The cleavage of C5 protein, by the C5 convertase during complement activation, generates C5a and C5b. The largest fragment, C5b, binds to the surface of microorganisms or non-self antigens and begins the assembling of the membrane attack complex (MAC), which in turn creates a pore in the microbial or foreign cell membrane, leading to their death [54]. The smallest fragment, C5a, is a 11-KDa glycoprotein, with potent inflammatory properties [21, 91]. The biological activities of C5a depend on the target cell and include: 1) vasodilation and enhancement of vascular permeability [8, 31, 73]; 2) smooth muscle contraction [8, 14, 31]: 3) chemotaxis [11, 36, 74] and degranulation of inflammatory cells [11, 35, 81]: 4) enhancement of the respiratory burst and consequent generation of reactive oxygen species in leukocytes [15, 29, 98]; 5) delayed neutrophil apoptosis; [61] 6) up-regulation of P-selectin expression from endothelial cells [22]; and 7) up-regulation of β_2 integrin and shedding of L-selectin adhesion molecules from granulocytes [45]. Other important functions of C5a are the induction and/or release of inflammatory cytokines such as interleukin (IL)-1 [58, 59, 71], IL-6 [2, 66, 72], IL-8 [19] and tumor necrosis factor (TNF) [59, 71] from neutrophils, mononuclear and endothelial cells. In addition, C5a exerts pro-

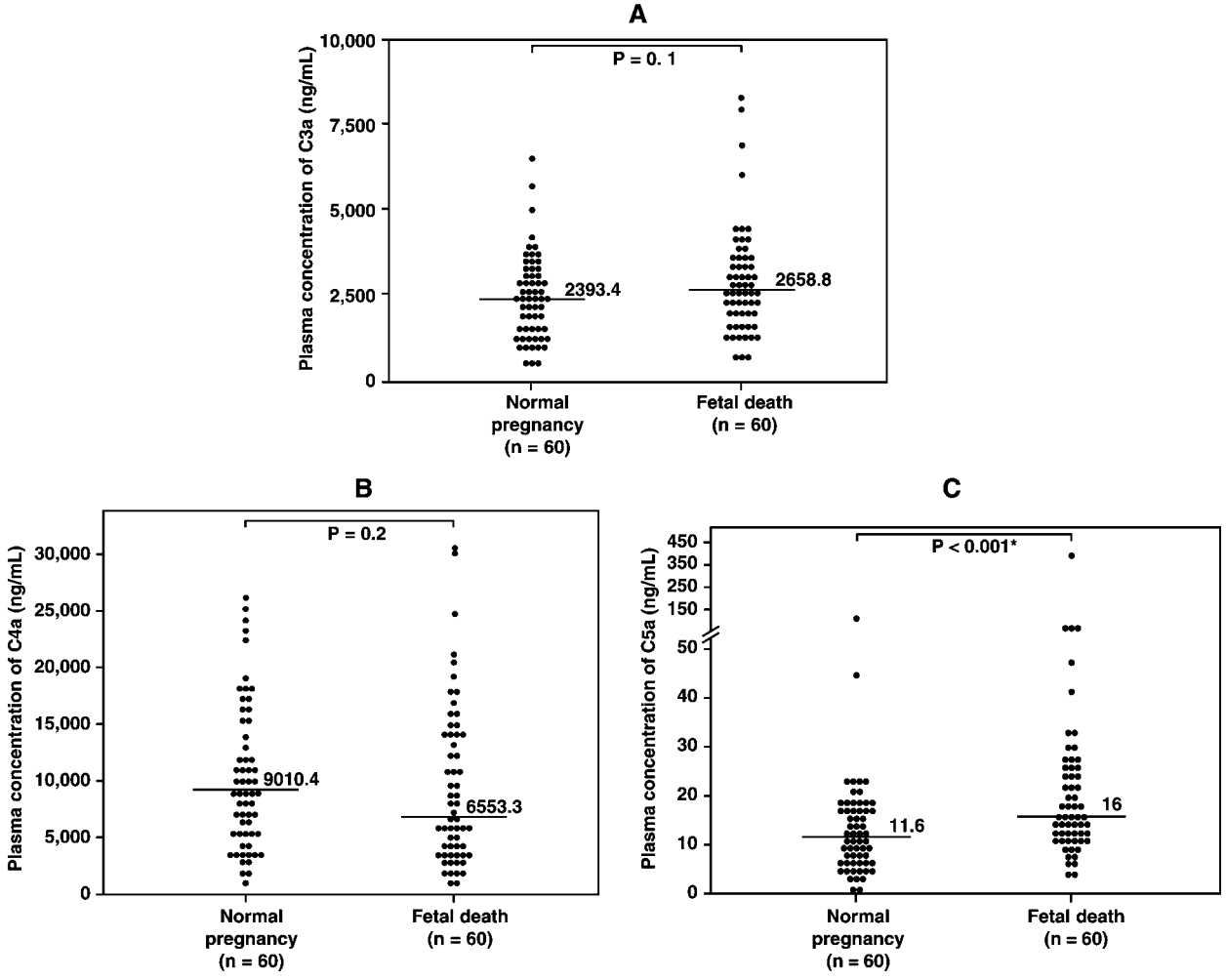

Figure 1 Plasma anaphylatoxin concentrations of normal pregnant women and patients with fetal death. **A**, No significant difference was observed in the median plasma C3a concentration between women with normal pregnancy and those with fetal death [median 2393.4 ng/mL (range 557.9–6642.7) vs. median 2658.8 ng/mL (range 612–8202.9); P = 0.1]. **B**, Similarly, there was no difference in the median plasma C4a concentration between women with normal pregnancy and those with fetal death [median 9010.4 ng/mL (range 850.7–27850) vs. median 6553.3 ng/mL (range 943.1–30020); P = 0.2]. **C**, In contrast, patients with fetal death had a higher median plasma C5a concentration than normal pregnant women [median 16 ng/mL (range 4.5–402.5) vs. median 11.6 ng/mL (range 1.2–87.1); P < 0.001].

coagulant effects [48] by promoting von-Willebrand factor and tissue factor secretion from endothelial cells [22, 44]. In turn, thrombin and kallikrein can activate C5 and generate C5a [30, 88, 94].

All complement pathways (the classic, alternative and mannose binding lectin) converge in the activation of C3 protein [89, 90]. The cleavage of C3 generates C3a and C3b. The latter acts as an opsonin and is part of the C5 convertase complex [54]. C3a biological effects are predominantly on mast cells and eosinophils, and include: 1) chemotaxis [11, 36]; 2) granule release [11, 81]; 3) expression and shedding of adhesion molecules (β2 integrin and L-selectin) [45]; and 4) increased oxidative burst in neutrophils and eosinophils [16, 17]. In addition, recent studies have attributed immunomodulatory effects to C3a, as this molecule enhances mRNA and protein expression for IL-1, IL-6 and TNF-alpha in isolated adherent mononuclear cells after LPS stimulation [79, 80]. C4a is the weakest of all anaphylatoxins inducing vascular permeability and smooth muscle contraction [31]. On the other hand, it has been suggested that C4a may modulate the inflammatory response since it can inhibit monocyte chemotaxis [85]. C5a generation may occur without sequential activation of the complement system [43, 86, 87, 92, 94]. Indeed, while conducting our study we found that fetal death was associated with higher maternal plasma concentrations of C5a without concomitant increase in C3a and C4a.

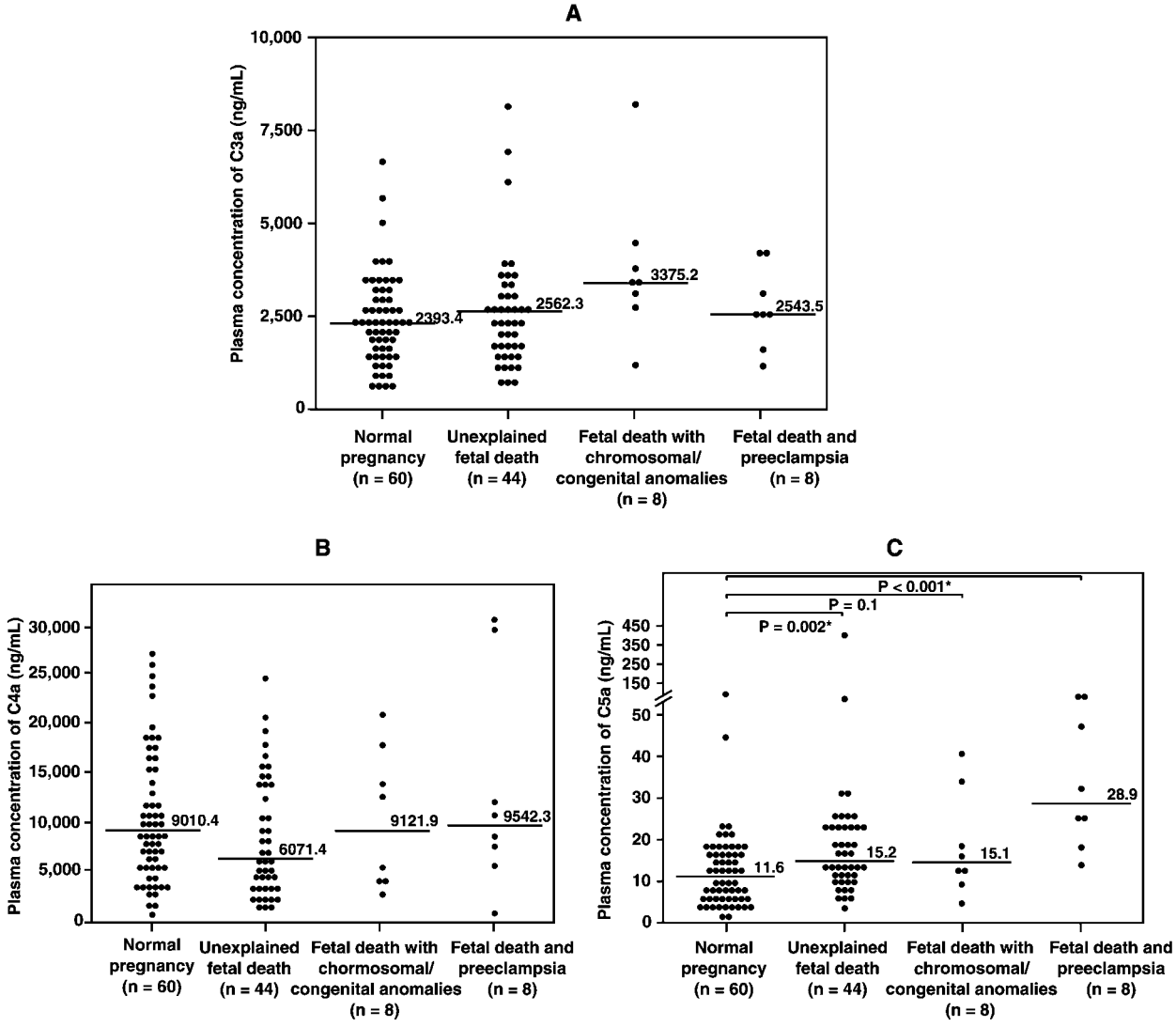

Figure 2 Plasma anaphylatoxin concentrations in the study groups. **A, B**, No differences were observed in the median plasma C3a and C4a concentrations among the study groups (Kruskal Wallis P = 0.1 and P = 0.3, respectively). **C,** In contrast, the plasma C5a concentration differed among the study groups (Kruskal Wallis P < 0.001). Post-hoc analysis showed that patients with unexplained fetal death and those associated with preeclampsia had a higher median plasma C5a concentration than normal pregnant women [unexplained fetal death: median 15.2 ng/mL (range 4.6–402.5), fetal death associated with preeclampsia: median 28.9 ng/mL (range 14.3–65.6) vs. normal pregnancy: median 11.6 ng/mL (range 1.2–87.1); P = 0.002 and < 0.001, respectively]. However, patients with fetal death associated with preeclampsia had a higher median C5a concentration than those with unexplained fetal death (P = 0.003). No significant difference was observed in plasma C5a concentration between fetal death with congenital anomalies [median 15.1 ng/mL (range 4.5–40.7)] and the other study groups.

Complement activation in normal human pregnancy

A recent study has demonstrated higher plasma concentrations of anaphylatoxins (C3a, C4a and C5a) in normal pregnant women than in non pregnant women in the secretory phase of the menstrual cycle [64]. The specific mechanisms responsible for this activation are unknown. We and others have proposed that complement activation is part of a compensatory state of the innate immune response during pregnancy, when the adaptive immune response is suppressed [67, 68] presumably to allow tolerance of the fetal semi-allograft. Enhanced innate immunity would protect the mother against infection.

How are trophoblast and the fetus protected against maternal complement activation during normal pregnancy? A role for complement regulatory proteins

Syncytiotrophoblast is in direct contact with maternal blood, which contains the entire set of complement proteins and neutrophils capable of producing C5a. Yet,

even when there is deposition of complement components in the chorionic villi and trophoblast basal membrane [20, 75, 83], under normal circumstances this tissue is not damaged by complement [82]. This has been attributed to the presence of membrane bound complement regulatory proteins which are expressed by cytotrophoblast and syncytiotrophoblast, including: 1) decay accelerating factor (DAF/CD55) [39, 40, 42, 82]; 2) membrane cofactor protein (MCP/CD46) [39, 42, 82]; and 3) protectin (CD59) [39, 82]. *In vitro* studies have shown that monoclonal antibodies against CD46 and CD59 increased the susceptibility of syncytiotrophoblast to complement lysis [82]. The importance of complement regulatory proteins has also been demonstrated in animal experiments. Indeed, embryos derived from Crry-deficient mice are not viable. In contrast, embryos derived from mice deficient in both Crry and C3 (double knockout for Crry-/- and C3-/-) are viable, suggesting that lethality is due to the inability to suppress spontaneous complement activation [99].

A possible role for complement activation in embryonic/fetal death

Previous studies have reported that both women with recurrent spontaneous abortion and primiparous women with first trimester pregnancy loss have complement activation, as suggested by a reduction in serum complement hemolytic activity, as well as C3 and factor B concentrations [9, 84]. However, decreased CH50 activity and low C3 concentrations can also result from decreased synthesis of complement components [1].

APS and other thrombophilic states (deficiency of antithrombin, factor V Leiden, protein S, protein C, or prothrombin mutations) have been proposed as possible causes of 2nd and 3rd trimester fetal death [3, 33, 60, 62, 63, 70]. Complement has been implicated in the mechanism of embryonic death in APS [25, 27, 38, 69]. Evidence in favor of this view is derived from animal experiments where: 1) administration of IgG containing phospholipid antibodies induced fetal resorption; and 2) monoclonal antibodies against C3 prevented fetal death and limited C3 deposition at the maternal fetal interface [27, 38]. Similar observations were described with blockage of C5a and its receptor (C5aR) [25]. The authors proposed that complement activation by phospholipid antibodies against the placenta generates anaphylatoxins (mainly C5a) that would attract and activate white blood cells and stimulate the release of proinflammatory mediators (including C3 and properdin), leading to placental injury and fetal death. Moreover, secretion of C3 and properdin would activate the alternative pathway, and neutrophils would also generate more C5a.

It is noteworthy that heparin prevented the effects of complement on trophoblast cells in culture, the deposition of C3 in the decidua as assessed by immunohistochemistry, and fetal death in an animal model of APS [26]. This protective effect of heparin has been attributed to its ability to block complement activity [47, 57, 93], rather than its anticoagulant effects [26].

Murine studies have shown the participation of complement activation in T-cell mediated fetal allograft rejection [53]. Indeed, Mellor et al. [53] demonstrated that blockage of IDO activity in pregnant mice deficient in $CD4^+$ T and B cells resulted in fetal rejection, extensive complement C3 deposition and inflammatory cell infiltration at the maternal-fetal interface despite an adequate Crry expression [53]. The expression of IDO in syncytiotrophoblast has been proposed to protect the allogenic conceptus from maternal T cell immunity, because this enzyme degrades tryptophan, an essential amino acid for T cell metabolism [46, 55]. Thus, the authors proposed a $CD8^+$ T cell dependent mechanism of complement activation and suggested that properdin released by maternal T cells induces complement activation [53].

Maternal/fetal infections have been implicated in the genesis of fetal death [24, 28]. However, the identification of microorganisms by standard culture-dependent methods have limitations [100]. We have previously reported that unexplained fetal death is associated with changes in the maternal adaptive immunity consistent with infection or prior antigenic exposure [5]. Indeed, women with unexplained fetal death had a higher percentage of "memory-like" T cells and a lower percentage of "naive-like" T cells than those with uncomplicated pregnancies, as determined by flow cytometry [5]. Thus, it is possible that a sub-clinical maternal and/or fetal infection may account for the high maternal plasma concentration of C5a among cases with unexplained intrauterine death. Evidence in favor of this likelihood is that maternal infection during pregnancy is associated with activation of granulocytes and monocytes [56], which in turn can release proteolytic enzymes (elastase and other serine protease) that are able to cleave C5 [43, 86, 87, 92]. Indeed, acute maternal infection during pregnancy is associated with high maternal plasma C5a concentration [76].

Collectively, the observations reported herein implicate complement activation in the pathophysiology of fetal death even in the absence of APS. It is tempting to postulate that an imbalance between complement activation and complement inhibition (complement regulatory proteins) may account for fetal death in some cases. The possibility that infection, antiphospholipid antibodies or T cells could induce excessive generation of C5a leading to fetal death should be considered.

The observation that maternal plasma concentration of C5a is high in the group of fetal deaths associated with preeclampsia is consistent with previous reports that activation of the complement system (with higher plasma C5a concentration) is present in preeclamptic patients [34, 65]. These findings provide additional evidence that preeclampsia is associated with maternal systemic

inflammation, which may be more apparent in the context of fetal death.

Future studies

Our study addresses the possible role of maternal plasma anaphylatoxins in fetal death. However, an evaluation of the balance between complement activation and inhibition in pregnancies complicated with fetal death would require the study of the changes in complement regulatory proteins in the trophoblast. In addition, longitudinal observations would be necessary to establish if the change in the maternal plasma concentration of C5a reported herein precedes fetal death or is a consequence of it.

In conclusion, maternal plasma concentrations of C5a are higher in women with fetal death than in those with normal pregnancies.

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