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Upregulation and release of soluble fms-like tyrosine kinase receptor 1 (sFLT1) mediated by complement activation in human syncytiotrophoblast cells

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Abstract

PROBLEM: Antiangiogenic molecule sFLT1 released from trophoblast cells is associated with pregnancy specific hypertensive disorder preeclampsia. Cause of elevated sFLT1 in preeclampsia patients is not well understood. Despite evidence of excess systemic and placental complement activation in preeclampsia patients its role in pathophysiology is not clear. If the complement activation plays a role in upregulation and secretion of sFLT1 is not known.

METHOD OF STUDY: Human trophoblast cells were isolated from term placentas and allowed to syncytialize. Complement was activated in vitro at sub-lethal levels on syncytiotrophoblast cells. Effect of complement activation on expression and release of sFLT1 was assessed by comparing its levels in these cells with and without complement activation.

RESULTS: Sub-lethal level of complement activation on syncytialized human trophoblast cells induced upregulation of sFLT1 mRNA and protein. Complement also induced secretion of sFLT1 in a manner depending on degree of activation. Anaphylatoxins C3a induced upregulation but not the release of sFLT1. Release of terminal membrane attack complex (MAC) was associated with sFLT1 secretion.

CONCLUSION: Complement activation plays a major role in both the expression and secretion of sFLT1 from syncytial trophoblast cells. The terminal MAC complex is involved in its secretion. Increased levels of sFLT1 in preeclampsia patients may be due to complement induced upregulation and secretion.

Keywords

Complement; syncytiotrophoblast; sFLT1; angiogenesis; Preeclampsia; Anaphylatoxins

1 INTRODUCTION

The circulating levels of antiangiogenic molecules such as soluble fms-like tyrosine kinase receptor 1 (sFLT1) and soluble endoglin have been shown to be significantly elevated in

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preeclampsia patients prior to the appearance of symptoms.^{1,2} The levels of these antiangiogenic molecules were correlated with the severity of the disease. Preeclampsia is a hypertensive disorder that affects about 3–5% of pregnancies with adverse fetal-maternal outcomes and it is a leading cause of maternal mortality. Most often diagnosed in the third trimester, other clinical manifestations of preeclampsia also include proteinuria, decreased kidney function, and elevated liver enzymes. Often preeclampsia is also associated with fetal growth restriction and preterm delivery. Preeclampsia also may lead to eclampsia and maternal mortality. Several risk factors such as family history, multiple gestation, nulliparity, maternal age, obesity, and diabetes etc. have been known to be associated with preeclampsia. Although several mechanisms such as hypoxia-oxidative stress, spiral artery remodeling, trophoblast differentiation etc. have been proposed to be involved in the pathology, role of sFLT1 is now well recognized.

Placenta is an important source of sFLT1 molecule during pregnancy and most of the sFLT1 immunoreactivity in human placentas has been shown to be associated with syncytiotrophoblast cells.³ Serum levels of sFLT1 start to decline immediately after delivery supporting its placental origin. An alternative splicing of FLT1 mRNA comprising of the extracellular domain of FLT1 produces sFLT1.⁴ Placental growth factor (PLGF) and vascular endothelial growth factor (VEGF), two important factors necessary for successful pregnancy utilize FLT1 as receptor.^{5,6} Circulating sFLT1 antagonizes PLGF and VEGF by preventing their binding to FLT1 and thus interfering with their biological functions.⁷ Several isoforms of sFLT1 (145Kd, 100Kd and 60Kd) in placenta and maternal plasma have been identified recently and shown to be elevated in preeclampsia patients.⁸

Semi-allogenic nature of fetus is long speculated to be an immunological challenge to the maternal immune system. Therefore, impaired maternal immune tolerance of the fetus has been proposed as a major trigger for many pregnancy associated diseases including preeclampsia.⁹ Systemic levels of complement activation products were higher in pregnant women compared to non-pregnant women which were further elevated in the circulation of preeclampsia patients.¹⁰ More importantly, the levels of these complement split products were elevated in the maternal circulation preceding the clinical disease indicating a causal relation to preeclampsia.^{11,12} In addition, higher amounts of complement split product C4d was found to be deposited on placentas from preeclampsia patients compared to those from normal pregnancy¹³ suggesting that placenta may be the origin for elevated systemic split products of complement activation found in preeclampsia patients.

The complement cascade consists of more than 30 proteins with most of them in the inactive form. Complement is activated through classical (antigen-antibody mediated) or lectin (cell surface carbohydrates) or alternative (de novo/loop with classical activation) pathways. Classical and lectin pathways start with the cleavage of serum protein C4 into C4b and C4a followed by covalent attachment of C4b to nearby cell surface. Alternative pathway initiation involves factor D mediated splitting of serum protein factor B. The central event in all three pathways is the formation of C3 convertase which in turn cleaves serum protein C3 into C3a and C3b. C3b covalently attaches to nearby cell surface and forms C5 convertase. Serum protein C5 is then split into C5a and C5b by C5 convertase. C5b again attached to cell surface binds to serum proteins C6, C7 and C8 to form a membrane pore like complex.

This complex is then reinforced by the insertion of one or more C9 molecules resulting in the generation of final product, the membrane attack complex (MAC). The split products C3a and C5a are released in to circulation. These products are anaphylotoxins capable of exerting biological functions through their receptors in various types of cells. Insertion of MAC in to cell membrane results in cell lysis due to its hydrophilic pore. However, nucleated eukaryotic cells resist MAC mediated lysis to variable extent by actively removing MAC lesions from their surface. But sub-lethal levels of MAC have been shown to activate a variety of signaling pathways in several cell types.

Although it is well known that pregnancy is associated with elevated systemic complement activation products originated in placenta and they are further elevated in preeclampsia patients, the effect of complement activation on human trophoblast cells in terms of generating antiangiogenic milieu is not well understood. In this study using human trophoblast cells isolated from placentas of normotensive pregnancies we show that *in vitro* complement activation upregulated *sFLT1* mRNA. Further, we also show that complement activation induced the secretion of sFLT1 in a manner dependent on the degree of activation and the secretion of sFLT1 was correlated with shedding of MAC from these cells.

2 METHODS

2.1 Human placentas

Term placentas were obtained from 5 normotensive pregnant women immediately after scheduled cesarean deliveries at Baylor College of Medicine and University of Texas Medical Branch. The study protocol was approved by Institutional Review Boards. Informed consent was obtained from the patients before placenta collection. Placentas were transferred sterile to the laboratory in saline and immediately washed thoroughly to remove blood. Villi were carefully scraped out to remove them from accompanying vessels and connective tissue, rinsed with saline and then minced in HBSS for trophoblast cell isolation. For proximity ligation assay (PLA), villous tissue was frozen in optimum cutting temperature (OCT) blocks and later thin sections (5µm) were made using a cryotome.

2.2 Trophoblast cell isolation

Trophoblast cells from term placentas were isolated as described earlier.^{14,15} Briefly, every 50g wet tissue was digested with trypsin (2.5%) in HBSS containing HEPES for 20 minutes at 37°C with gentle and constant stirring in a total volume of 150 mL. DNase (1.2 mL of 10×) was then added and the incubation was continued for another 10 minutes. The extract free of debris was then passed through a cell strainer and collected in FBS containing tubes. The remaining tissues were then digested two more times and similarly collected in FBS containing tubes. These tubes were centrifuged for 6 minutes at 1200g. Pellets were re-suspended while the supernatants were discarded. The suspended pellet was then layered on top of 60–30% percoll gradient and centrifuged for 20 minutes at 1200g. The middle portion of the gradient containing trophoblast cells was then carefully removed, washed in DMEM. Cells were then counted and plated in 6-well cell culture plates with a density of 1.5 million cells/plate in DMEM containing 10% FBS at 37°C, 5% CO₂ and 95% air. After 72 hours,

syncytialization was checked by examining the production of human chorionic gonadotropin.

2.3 Complement activation

Pooled normal human serum (NHS) was used as source of complement for in vitro complement activation on syncytialized trophoblast cells. Antibody pre-sensitization or zymosan (Complement Technology, Inc. USA) was used as trigger to accelerate the complement activation. For antibody presensitization trophoblast cells were incubated in DMEM (without NHS or FBS) containing required amounts of anti-HLAG antibody (Santa Cruz Biotechnologies, USA) for one hour and then washed twice with fresh DMEM to remove unbound antibodies. Zymosan at required level was first suspended in VBS⁺⁺ buffer (Complement Technology, Inc. USA), NHS was then diluted in DMEM to the required dilution (10%). Cells were washed 6 times to remove traces of FBS, diluted serum was added and then zymosan was added. After 24 hours, culture supernatants were collected, aliquoted and kept frozen at -80°C until used for assays. Cells were washed thoroughly 6 times using ice cold PBS and then harvested in cell lysis buffer (Cell Signaling Technology, USA), centrifuged at 1200g for 10 minutes at 4°C to pellet cell debris. Cell lysates were then collected, aliquoted and kept frozen at -80°C until further use. For RNA isolation cells were harvested according to the manual provided with RNeasy mini kit (Qiagen, USA)

2.4 Immunoassays

sFLT1 was quantified using VEGFR1 ELISA kit (Reddot Biotech, Canada). MAC quantification was done by using MAC ELISA kit (MyBiosource, USA).

2.5 RT-qPCR

FLT1 mRNA levels were measured by qRT-PCR using SYBR green (Bio-Rad, USA) as described earlier.¹⁶ Total RNA was isolated from trophoblast cells using the RNeasy mini kit. cDNA was synthesized using SuperScript III one step RT-PCR system with platinum Taq DNA polymerase (Life Technologies, USA). The primer sequences used were, forward 5'-TTGGGACTGTGGGAAGAAAC-3' and reverse 5'-TTGGAGATCCGAGAGAAAACA-3'. β -actin was used as internal control.

2.6 Western analysis

Cell lysates containing 10 μg protein were electrophoresed on 5–20% (w/v) polyacrylamide gradient gels (Novex, Life Technologies) and transferred on to Immune-Blot PVDF membranes (Bio-Rad). Expression of sFLT1 was probed with goat polyclonal human anti-FLT1 antibody (R&D system, USA). A secondary antibody rabbit anti-goat IgG-HRP (Southern Biotech, USA) and pierce ECL Western blotting substrate kit (Thermo Scientific, USA) were used to develop the membranes. The Western bands were visualized using Odyssey IR imaging system (LI-COR Biosciences, USA).

2.7 Proximity Ligation Assay

PLA was performed according to the instructions provided by the manufacturer (Olink Biosciences, Uppsala, Sweden). Briefly, villous tissue sections were incubated with primary

antibodies for goat polyclonal human anti-C9 and Rabbit Anti-Human SC5b-9 Neoantigen (Complement Technologies, USA) diluted 1:500 in blocking solution for 90 minutes at 37°C in a humid chamber. A negative control was prepared by incubating the cells in blocking solution with isotype controlled IgGs. After the incubation with primary antibodies, tissue sections were washed with buffer A (0.01 M Tris, 0.15 M NaCl, and 0.05% Tween-20, pH 7.4) for 2×10 minutes and then incubated with goat plus and rabbit minus PLA probes (1:5 diluted in antibody diluents) for 60 minutes at 37°C. The total reaction mixture in all the steps was 30µl/section. Sections were again washed with buffer A for 2×5 minutes and incubated with ligation mixture (1:5 diluted in molecular grade water) for 30 minutes at 37°C. Sections were washed with buffer A for 2×2 minutes and incubated with amplification mixture (1:5 diluted in molecular grade water) for 100 minutes at 37°C. Sections were then washed twice with buffer B (0.2 M Tris, 0.1 M NaCl, pH 7.5) for 10 minutes each and rinsed with 0.01× buffer B, allowed to dry at room temperature in the dark. Slides were then mounted with cover slips using duolink II mounting medium and the images were observed under fluorescence microscope (Olympus, U-TV1 X) under 20× objective. Images were processed and red spots were counted using Image-Pro Plus software (MediaCybernetics, U.S.A).

2.8 Statistical analysis

The data are presented as mean $\pm$ SEM. Data obtained from paired experiments where cells isolated from same placenta were treated with different zymosan or anti-HLAG antibody concentrations were analyzed using Wilcoxon's test. Data from triplicate experiments for more than two groups were analyzed by ANOVA followed by Tukey's multiple comparison tests. Student t-test was used when two groups were compared followed by Mann-Whitney test. A *p* value of less than 0.05 was considered to be significant.

3. RESULTS

3.1 Complement activation upregulates *sFLT1* levels in human syncytial trophoblast cells

Isolated trophoblast cells were cultured for 72 hours to undergo spontaneous syncytialization. An average of 94% purity was obtained for trophoblast cells with the current isolation method as assessed by counting cytokeratin-7 positive cells (Fig1 A). We used 10% pooled normal human serum (NHS) as source of complement proteins because at this dilution the complement activation generated non-lethal doses of MAC on human neutrophils.¹⁷ We used zymosan which is a commonly used yeast cell wall derivative to trigger the complement activation via all three pathways of complement cascade¹⁸. Initially we optimized the zymosan concentrations to attain sub lethal levels of complement activation at different degrees. Deposition and release of MAC as assessed by immunostaining and ELISA respectively confirmed that 0.01, 0.1 and 0.5 % zymosan in culture media activated complement to different degrees (Fig1 B-D). We then examined cytotoxicity of complement activation at these levels on syncytiotrophoblast cells by LDH release assay (Fig1 E). The % cytotoxicity was 0.79 ± 0.06 , 1.82 ± 1.05 and 3.98 ± 1.67 upon complement activation using 0.01, 0.1 and 0.5 % zymosan respectively indicating that complement activation was sub lethal.

To test the effect of complement activation on expression of *sFLT1*, mRNA levels were measured by RT-qPCR at 24 hours. Complement activation using 0.01% zymosan significantly increased *sFLT1* mRNA levels (2 ± 0.09 fold, $p=0.028$) compared to NHS alone without zymosan and C3/C4 depleted NHS with 0.01% zymosan control groups (Fig2 A). At protein level, expression of all three isoforms of sFLT1 (145Kd, 100Kd and 60Kd) increased upon complement activation using 0.01% zymosan compared to zymosan negative and NHS negative control groups (Fig2 B). Immunostaining for sFLT1 in syncytiotrophoblast cells confirmed that complement activation significantly increased sFLT1 protein levels compared to no complement activation (1.97 ± 0.3 fold, $p=0.03$). Immunostaining further showed co-localization of sFLT1 staining with cytokeratin-7 staining (Fig2 C & D) indicating that complement activation increased sFLT1 protein levels in cytokeratin positive syncytiotrophoblast cells.

3.2 Complement activation induced secretion of sFLT1

As degree of complement activation depends on concentrations of effector and regulator proteins and their structure-function status; we sought to test the effect of degree of complement activation on sFLT1 protein levels in trophoblast cells. Western blot analysis of cell lysates (Fig3 A) followed by protein band density analysis showed that complement activation with all three concentrations of zymosan significantly increased 100 Kd sFLT1 protein levels in these cells (4.12 ± 0.22 , 2.9 ± 0.33 , 2.07 ± 0.17 -fold with 0.01 ($p<0.0001$), 0.1 ($p=0.0003$) and 0.5% ($p=0.037$) zymosan respectively). However, 100 Kd sFLT1 protein levels (Fig3 B) in cell lysates decreased dose dependently with increasing concentrations of zymosan (1.48 ± 0.3 -fold for 0.5 v/s 0.1, $p=0.06$; 2.04 ± 0.23 -fold for 0.5 v/s 0.01, $p=0.0003$; 1.4 ± 0.12 -fold for 0.1 v/s 0.01 % zymosan, $p=0.01$, respectively). Similar trend was observed in the expression levels of other two isoforms of sFLT1 (quantitation not shown). These results suggested that complement activation increased sFLT1 levels in a manner dependent on the degree of activation, however, as degree of activation increased cellular levels of sFLT1 proteins decreased. This led us to hypothesize that complement activation induced release of sFLT1 protein depending on the degree of activation.

To test this hypothesis, sFLT1 levels in cell culture media were measured by ELISA (Fig3 C). Complement activation with all three concentrations of zymosan significantly increased sFLT1 protein levels in culture media compared to no complement activation (0.21 ± 0.03 ng/ml, 1.12 ± 0.62 ng/ml, 1.7 ± 0.68 ng/ml and 2.69 ± 1.2 ng/ml with 0, 0.01, 0.1 and 0.5% zymosan respectively, $p=0.015$). Further, the increase of sFLT1 levels in culture media was dependent on the degree of complement activation. Together, these data showed that complement induced the secretion of sFLT1 from syncytialized human trophoblast cells depending on the degree of activation.

Zymosan triggers classical complement activation in the fluid phase by binding to its natural antibodies present in NHS. To test if complement activation by antigen-antibody complex formed on the surface of syncytiotrophoblast cells induces same response as zymosan in terms of upregulation and release of sFLT1 we examined the effect of complement activation triggered by presensitization of the cells with anti-HLAG antibodies. Initially we optimized antibody dose at sub saturation levels (Fig4 A) to attain MAC levels similar to that obtained

by zymosan (Fig4 B). RT-qPCR of *sFLT1* mRNA (Fig4 C) revealed that complement activation triggered by antibody presensitization significantly upregulated sFLT1 expression (1.3 ± 0.02 fold, $p=0.028$). ELISA of sFLT1 (Fig4 D) further showed that complement triggered by antibody presensitization induced the release of sFLT1 in to culture media in a manner dependent on degree of activation (0.12 ± 0.01 ng/ml, 0.79 ± 0.04 ng/ml and 1.5 ± 0.02 ng/ml with 0, 2.5 and 5 μ g antibodies respectively, $p=0.007$).

3.3 Higher levels of MAC deposited on placentas from preeclampsia patients

To test if complement activation is increased on placenta in preeclampsia patients we compared MAC levels on placentas from preeclampsia and normotensive pregnancies by performing PLA. As C9 is part of MAC complex, we used anti-C9 antibody and anti-MAC antibody that recognizes a neoepitope present only in the full MAC complex. Therefore, each fluorescent dot on the tissue represents a single MAC complex. The comparison between two groups was made by normalizing the number of dots in microscopic area to number of nuclei and expressed as dots/nucleus. The PLA (Fig 5A) followed by quantification of dots per nucleus revealed (Fig5 B) that significantly higher levels of MAC were deposited on placentas from preeclampsia patients compared to those from normotensive pregnancies (2.26 ± 0.92 and 0.59 ± 0.16 dots/nucleus respectively, $p=0.03$).

3.4 C3a induced upregulation but not the release of sFLT1

We partially addressed which components of complement cascade are involved in the sFLT1 upregulation and release. To this end, we tested if anaphylatoxin C3a induces upregulation and release of sFLT1 because trophoblast cells express its receptor C3aR. RT-qPCR (Fig6 A) revealed that treating cells with recombinant human C3a (2 μ g/ml, equivalent to amount produced by zymosan) significantly increased sFLT1 mRNA levels compared to no C3a (1.44 ± 0.06 fold, $p=0.0005$). However, ELISA data (Fig6 B) indicated that C3a treatment failed to induce the release of sFLT1 in to culture media. We further analyzed if MAC level correlate with sFLT1 release (Fig7 A & B). The results suggested that release of MAC from these cells but not the cellular levels positively correlated with sFLT1 levels in culture media Pearson $r=0.569$, $p=0.02$).

4 DISCUSSION

To our knowledge this is the first report of a direct effect of complement cascade activation on human trophoblast cells in terms of inducing the upregulation and secretion of sFLT1. The data presented here suggest that sub lethal levels of complement activation on syncytialized human trophoblast cells induced upregulation as well as secretion of sFLT1. The data also suggest that the anaphylatoxin C3a induced upregulation of sFLT1 in these cells but not its secretion. On the other hand, terminal MAC shedding from trophoblast cells correlated with sFLT1 secretion.

Our finding that complement activation induced upregulation and secretion of sFLT1 in human syncytial trophoblast cells is important in the context of human studies showing elevated levels of sFLT1 in maternal circulation prior to the onset of preeclampsia symptoms.^{21,22} Mechanistically sFLT1 has been shown to dysregulate TGF- β and nitric

oxide signaling and thus affect the vascular functions responsible for maternal symptoms of preeclampsia.²³ Now it is well accepted that placenta is the major source of sFLT1²⁴ and delivery of placenta leads to immediate decrease in serum sFLT1 levels.²¹ We have shown that both zymosan and antibody presensitization mediated complement activation induced upregulation and release of sFLT1 in syncytiotrophoblast cells. Zymosan activates classical pathway in culture medium via antigen-antibody complex formation due to the presence of natural antibodies against zymosan in NHS. This is similar to complement activation by autoantibodies and thus could explain increased frequency of preeclampsia in women with autoimmune diseases such as antiphospholipid syndrome. Because of its semi-allogenic nature placenta could trigger an immune response from the maternal system and in fact it has been shown that complement activation products such as C3a and C5a were elevated in the maternal circulation in pregnant women compared to non-pregnant women.²⁵ Complement activation in normal pregnancy may be due to the direct contact of syncytiotrophoblast cells with maternal blood and thus exposure of paternal antigens to the maternal complement. Antibody presensitization mimics exposure of paternal antigens to maternal blood and subsequent formation of antigen-antibody complexes and thus could explain complement activation on syncytiotrophoblast cells in normal pregnancy. However, normal pregnancy appears to be well protected from a chronic complement activation due to higher levels of expression of cell surface complement regulators on syncytiotrophoblast cells.^{26,27} In preeclampsia this normal protection appears to be compromised as loss of function mutations in surface regulators such as CD46 have been shown to be associated with preeclampsia.²⁸ Our data showing significantly increased MAC levels on placentas obtained from preeclampsia patients is consistent with these reports.

Exposure of human cytotrophoblast cells in vitro to low oxygen levels (hypoxia) induces upregulation and secretion of sFLT1.^{29,30} These in vitro findings were extrapolated to in vivo condition leading to the presumption that hypoxia is the primary driver of antiangiogenic milieu. However, role of hypoxia in the pathogenesis of preeclampsia is unclear.³¹ Exposure of pregnant rodents to hypoxia increased the invasion of trophoblast cells in to maternal uterine spiral arteries.³² Contrary to this, reduced trophoblast invasion and the subsequent defective uterine spiral artery remodeling have been consistently postulated to be central to preeclampsia pathogenesis.^{33,34} Although oxygen tension in first trimester human placenta is low (hypoxic), it is now well accepted as normal physiological state which is required for normal placental development.³⁵ In vivo studies relating placental hypoxia to antiangiogenic milieu and eventually preeclampsia heavily rely on increased expression of hypoxia response element HIF1 α . Recent data suggest that HIF1 α was also reactive to autoantibodies such as anti-angiotensin II type I receptor and various stressors through stress kinases.^{36, 35} Further, HIF1 α could modulate angiogenic factors independent of oxygen levels.³⁵ Nevertheless, immunological factors may be upstream to the development of placental hypoxia.³⁷ Therefore, we believe that complement mediated upregulation and secretion of sFLT-1 could be independent of hypoxia mediated generation of antiangiogenic milieu. Further research is required to determine the relative contributions or interrelationships between hypoxia mediated and complement mediated antiangiogenic condition in the pathophysiology of preeclampsia.

While our findings in the present study are novel and exciting, there are certain limitations. It is not possible to address the early first trimester events of placental development using syncytialized trophoblast cells derived from term placenta. We have not addressed the effect of complement activation on extra villous cytotrophoblast cells which play a major role in uterine artery remodeling due to our limitations in obtaining first trimester placental tissues. Nonetheless, our data suggest that the source of sFLT1 which start to rise in preeclampsia patients at gestation weeks 21–24³⁸ may be from syncytial trophoblast cells and this increase may be mediated by complement activation. Our experimental design using syncytialized trophoblast cells is likely very close to in vivo situation because during human placentation syncytial layer is fully formed and maternal blood circulation to intervillous space is established as early as 12–13 weeks of gestation.³⁹

The most intriguing question is how an arm of immune system that was evolutionarily designed and conserved to kill foreign cells induced upregulation and secretion of angiogenesis related molecule sFLT1. Following recent developments on non-canonical functions it is not surprising that complement cascade can induce up regulation of gene expression. Complement activation in the circulation as an immune response to infection leads to substantial defense partly through lysis of microbial cells. However, nucleated eukaryotic cells express complement regulators on the cell surface in addition to circulating regulators which are recruited on to the cell surface in response to complement activation in the vicinity. Together, these regulators control the explosive propagation of the cascade and thus protect self-cells from MAC mediated lysis.⁴⁰ On the other hand anaphylatoxins and MAC and its intermediates generated at sub-lethal levels of complement activation induce cell activation in different types of cells in a cell specific manner.^{17,41,42} Insertion of MAC into plasma membrane increased cytosolic Ca^{2+} and PKC activities in Ehrlich cells.^{43,44} The MAC and intermediates also induced lipid derived signal molecules such as 1,2-diacylglycerol and ceramide in human B cell line JY25.⁴⁵ Upstream anaphylatoxins as well as terminal MAC and its intermediates activated several G proteins and kinases such as Ras, Raf-1, ERK1, PI3K/Akt, JNK1 and MAPK in various cells such as JY25, human aortic smooth muscle cells and macrophages.^{46,47,48,49} Interestingly, obesity related hormone resistin up-regulated FLT1 through the activation of ERK1 and MAPK in human endothelial cells.⁵⁰ In monocyte/macrophage cells, kinesin superfamily protein 4 upregulated the FLT1 via activation of PI3K/Akt signaling.⁵¹ Therefore, we believe that some of these signaling pathways could provide link between complement and sFLT1 up-regulation and secretion. Future research will be focused on the mechanism of signal transduction downstream to complement activation that results in the up-regulation and secretion of sFLT1 in human syncytialized trophoblast cells.

Our data suggested that anaphylatoxin C3a induced up regulation of sFLT1 but not its secretion from trophoblast cells indicating that other components of the cascade such as MAC may be important in the secretion of sFLT1. Nucleated eukaryotic cells physically remove potentially lytic MAC and its intermediate complexes from their surfaces without suffering loss of significant plasma membrane.¹⁷ The physical removal of MAC occurs by both endocytosis and exocytosis.⁵² It is possible that sFLT1 in trophoblast cells was secreted out as part of endo and/or exocytosis process of MAC removal. Our data indicating a good correlation between MAC levels and secreted sFLT1 levels is consistent with this hypothesis.

The effect of complement activation on sFLT1 secretion in vivo could be more robust than our in vitro studies as the Ca^{2+} levels used in our assays were limited. The removal of MAC from cell surface has been shown to be mediated by PKC activation and subsequent Ca^{2+} influx. Further, rate of elimination of MAC was governed by extracellular Ca^{2+} concentrations.⁴⁴ The Ca^{2+} concentrations in the media used in our assays (0.15mM) were substantially lower than the circulating levels of (Ca^{2+}) ⁵³ potentially limiting the rate of MAC removal.

Collectively, we have shown that complement activation induced upregulation and secretion of sFLT1 in human syncytiotrophoblast cells. Data presented here show that anaphylatoxins play a role in the upregulation of sFLT1 but not in the secretion. Future research will be focused on the mechanisms of upregulation and secretion of sFLT1. These findings open up several new avenues of research to find out the overall impact of complement activation on antiangiogenic milieu in terms of its effect on PlGF, VEGF and soluble endoglin.

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References

1. Levine RJ, Lam C, Qian C, Yu KF, Maynard SE, Sachs BP, Sibai BM, Epstein FH, Romero R, Thadhani R, Karumanchi SA: Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. *The New England journal of medicine* 2006;355:992–1005. [PubMed: 16957146]
2. Venkatesha S, Toporsian M, Lam C, Hanai J, Mammoto T, Kim YM, Bdolah Y, Lim KH, Yuan HT, Libermann TA, Stillman IE, Roberts D, D’Amore PA, Epstein FH, Sellke FW, Romero R, Sukhatme VP, Letarte M, Karumanchi SA: Soluble endoglin contributes to the pathogenesis of preeclampsia. *Nature medicine* 2006;12:642–649.
3. Buurma AJ, Penning ME, Prins F, Schutte JM, Bruijn JA, Wilhelmus S, Rajakumar A, Bloemenkamp KW, Karumanchi SA, Baelde HJ: Preeclampsia is associated with the presence of transcriptionally active placental fragments in the maternal lung. *Hypertension (Dallas, Tex : 1979)* 2013;62:608–613.
4. Kendall RL, Thomas KA: Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. *Proceedings of the National Academy of Sciences of the United States of America* 1993;90:10705–10709. [PubMed: 8248162]
5. Lynch AM, Salmon JE: Dysregulated complement activation as a common pathway of injury in preeclampsia and other pregnancy complications. *Placenta* 2010;31:561–567. [PubMed: 20427084]
6. Ferrara N, Gerber HP, LeCouter J: The biology of VEGF and its receptors. *Nature medicine* 2003;9:669–676.
7. He Y, Smith SK, Day KA, Clark DE, Licence DR, Charnock-Jones DS: Alternative splicing of vascular endothelial growth factor (VEGF)-R1 (FLT-1) pre-mRNA is important for the regulation of VEGF activity. *Molecular endocrinology (Baltimore, Md)* 1999;13:537–545.
8. Rajakumar A, Powers RW, Hubel CA, Shibata E, von Versen-Hoynck F, Plymire D, Jeyabalan A: Novel soluble Flt-1 isoforms in plasma and cultured placental explants from normotensive pregnant and preeclamptic women. *Placenta* 2009;30:25–34. [PubMed: 19010535]
9. Guleria I, Sayegh MH: Maternal acceptance of the fetus: true human tolerance. *Journal of immunology (Baltimore, Md : 1950)* 2007;178:3345–3351.
10. Derzsy Z, Prohaszka Z, Rigo J, Jr., Fust G, Molvarec A: Activation of the complement system in normal pregnancy and preeclampsia. *Molecular immunology* 2010;47:1500–1506. [PubMed: 20181396]

11. Lynch AM, Murphy JR, Byers T, Gibbs RS, Neville MC, Giclas PC, Salmon JE, Holers VM: Alternative complement pathway activation fragment Bb in early pregnancy as a predictor of preeclampsia. *American journal of obstetrics and gynecology* 2008;198:385.e381–389. [PubMed: 18221926]
12. Lynch AM, Eckel RH, Murphy JR, Gibbs RS, West NA, Giclas PC, Salmon JE, Holers VM: Prepregnancy obesity and complement system activation in early pregnancy and the subsequent development of preeclampsia. *American journal of obstetrics and gynecology* 2012;206:428.e421–428. [PubMed: 22542119]
13. Buurma A, Cohen D, Veraar K, Schonkeren D, Claas FH, Bruijn JA, Bloemenkamp KW, Baelde HJ: Preeclampsia is characterized by placental complement dysregulation. *Hypertension (Dallas, Tex : 1979)* 2012;60:1332–1337.
14. Kliman HJ, Nestler JE, Sermasi E, Sanger JM, Strauss JF, 3rd: Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. *Endocrinology* 1986;118:1567–1582. [PubMed: 3512258]
15. Green KE, Thota C, Hankins GD, Yallampalli C, Dong YL: Calcitonin gene-related peptide stimulates human villous trophoblast cell differentiation in vitro. *Molecular human reproduction* 2006;12:443–450. [PubMed: 16772432]
16. Ikeda T, Sun L, Tsuruoka N, Ishigaki Y, Yoshitomi Y, Yoshitake Y, Yonekura H: Hypoxia down-regulates sFlt-1 (sVEGFR-1) expression in human microvascular endothelial cells by a mechanism involving mRNA alternative processing. *The Biochemical journal* 2011;436:399–407. [PubMed: 21382012]
17. Morgan BP: Complement membrane attack on nucleated cells: resistance, recovery and non-lethal effects. *The Biochemical journal* 1989;264:1–14. [PubMed: 2690818]
18. Huber AR, Weiss SJ: Disruption of the subendothelial basement membrane during neutrophil diapedesis in an in vitro construct of a blood vessel wall. *The Journal of clinical investigation* 1989;83:1122–1136. [PubMed: 2703527]
19. Smith MC, Pensky J, Naff GB: Inhibition of zymosan-induced alternative complement pathway activation by concanavalin A. *Infection and immunity* 1982;38:1279–1284. [PubMed: 6924928]
20. Fearon DT, Austen KF: Activation of the alternative complement pathway due to resistance of zymosan-bound amplification convertase to endogenous regulatory mechanisms. *Proceedings of the National Academy of Sciences of the United States of America* 1977;74:1683–1687. [PubMed: 266208]
21. Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, Libermann TA, Morgan JP, Sellke FW, Stillman IE, Epstein FH, Sukhatme VP, Karumanchi SA: Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *The Journal of clinical investigation* 2003;111:649–658. [PubMed: 12618519]
22. Zeisler H, Llorba E, Chantraine F, Vatish M, Staff AC, Sennstrom M, Olovsson M, Brennecke SP, Stepan H, Allegranza D, Dilba P, Schoedl M, Hund M, Verlohren S: Predictive Value of the sFlt-1:PIGF Ratio in Women with Suspected Preeclampsia. *The New England journal of medicine* 2016;374:13–22. [PubMed: 26735990]
23. Powe CE, Levine RJ, Karumanchi SA: Preeclampsia, a disease of the maternal endothelium: the role of antiangiogenic factors and implications for later cardiovascular disease. *Circulation* 2011;123:2856–2869. [PubMed: 21690502]
24. Palmer KR, Tong S, Kaitu'u-Lino TJ: Placental-specific sFLT-1: role in pre-eclamptic pathophysiology and its translational possibilities for clinical prediction and diagnosis. *Molecular human reproduction* 2017;23:69–78. [PubMed: 27986932]
25. Richani K, Soto E, Romero R, Espinoza J, Chaiworapongsa T, Nien JK, Edwin S, Kim YM, Hong JS, Mazor M: Normal pregnancy is characterized by systemic activation of the complement system. *The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstet* 2005;17:239–245.
26. Houlihan JM, Biro PA, Fergar-Payne A, Simpson KL, Holmes CH: Evidence for the expression of non-HLA-A,-B,-C class I genes in the human fetal liver. *Journal of immunology (Baltimore, Md : 1950)* 1992;149:668–675.

27. Holmes CH, Simpson KL, Wainwright SD, Tate CG, Houlihan JM, Sawyer IH, Rogers IP, Spring FA, Anstee DJ, Tanner MJ: Preferential expression of the complement regulatory protein decay accelerating factor at the fetomaternal interface during human pregnancy. *Journal of immunology* (Baltimore, Md : 1950) 1990;144:3099–3105.
28. Salmon JE, Heuser C, Triebwasser M, Liszewski MK, Kavanagh D, Roumenina L, Branch DW, Goodship T, Fremeaux-Bacchi V, Atkinson JP: Mutations in complement regulatory proteins predispose to preeclampsia: a genetic analysis of the PROMISSE cohort. *PLoS medicine* 2011;8:e1001013. [PubMed: 21445332]
29. Nagamatsu T, Fujii T, Kusumi M, Zou L, Yamashita T, Osuga Y, Momoeda M, Kozuma S, Taketani Y: Cytotrophoblasts up-regulate soluble fms-like tyrosine kinase-1 expression under reduced oxygen: an implication for the placental vascular development and the pathophysiology of preeclampsia. *Endocrinology* 2004;145:4838–4845. [PubMed: 15284201]
30. Gerber HP, Condorelli F, Park J, Ferrara N: Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. *The Journal of biological chemistry* 1997;272:23659–23667. [PubMed: 9295307]
31. Charnock-Jones DS: Placental hypoxia, endoplasmic reticulum stress and maternal endothelial sensitisation by sFLT1 in pre-eclampsia. *Journal of reproductive immunology* 2016;114:81–85. [PubMed: 26228018]
32. Rosario GX, Konno T, Soares MJ: Maternal hypoxia activates endovascular trophoblast cell invasion. *Developmental biology* 2008;314:362–375. [PubMed: 18199431]
33. Goldman-Wohl D, Yagel S: Regulation of trophoblast invasion: from normal implantation to pre-eclampsia. *Molecular and cellular endocrinology* 2002;187:233–238. [PubMed: 11988332]
34. Reister F, Heyl W, Kaufmann P, Rath W: [Trophoblast invasion in pre-eclampsia]. *Zentralblatt fur Gynakologie* 1999;121:587–590. [PubMed: 10666868]
35. Cindrova-Davies T, van Patot MT, Gardner L, Jauniaux E, Burton GJ, Charnock-Jones DS: Energy status and HIF signalling in chorionic villi show no evidence of hypoxic stress during human early placental development. *Molecular human reproduction* 2015;21:296–308. [PubMed: 25391298]
36. Iriyama T, Wang W, Parchim NF, Song A, Blackwell SC, Sibai BM, Kellems RE, Xia Y: Hypoxia-independent upregulation of placental hypoxia inducible factor-1alpha gene expression contributes to the pathogenesis of preeclampsia. *Hypertension (Dallas, Tex : 1979)* 2015;65:1307–1315.
37. Karumanchi SA, Bdolah Y: Hypoxia and sFlt-1 in preeclampsia: the “chicken-and-egg” question. *Endocrinology* 2004;145:4835–4837. [PubMed: 15489315]
38. Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, Schisterman EF, Thadhani R, Sachs BP, Epstein FH, Sibai BM, Sukhatme VP, Karumanchi SA: Circulating angiogenic factors and the risk of preeclampsia. *The New England journal of medicine* 2004;350:672–683. [PubMed: 14764923]
39. Malassine A, Frendo JL, Evain-Brion D: A comparison of placental development and endocrine functions between the human and mouse model. *Human reproduction update* 2003;9:531–539. [PubMed: 14714590]
40. Zipfel PF, Skerka C: Complement regulators and inhibitory proteins. *Nature reviews Immunology* 2009;9:729–740.
41. Tegli CA, Cudrici C, Patel S, Trippe R, 3rd, Rus V, Niculescu F, Rus H: Membrane attack by complement: the assembly and biology of terminal complement complexes. *Immunologic research* 2011;51:45–60. [PubMed: 21850539]
42. Merle NS, Noe R, Halbwachs-Mecarelli L, Fremeaux-Bacchi V, Roumenina LT: Complement System Part II: Role in Immunity. *Frontiers in immunology* 2015;6:257. [PubMed: 26074922]
43. Carney DF, Lang TJ, Shin ML: Multiple signal messengers generated by terminal complement complexes and their role in terminal complement complex elimination. *Journal of immunology* (Baltimore, Md : 1950) 1990;145:623–629.
44. Carney DF, Hammer CH, Shin ML: Elimination of terminal complement complexes in the plasma membrane of nucleated cells: influence of extracellular Ca²⁺ and association with cellular Ca²⁺. *Journal of immunology* (Baltimore, Md : 1950) 1986;137:263–270.

45. Niculescu F, Rus H, Shin S, Lang T, Shin ML: Generation of diacylglycerol and ceramide during homologous complement activation. *Journal of immunology* (Baltimore, Md : 1950) 1993;150:214–224.
46. Niculescu F, Rus H, van Biesen T, Shin ML: Activation of Ras and mitogen-activated protein kinase pathway by terminal complement complexes is G protein dependent. *Journal of immunology* (Baltimore, Md : 1950) 1997;158:4405–4412.
47. Niculescu F, Badea T, Rus H: Sublytic C5b-9 induces proliferation of human aortic smooth muscle cells: role of mitogen activated protein kinase and phosphatidylinositol 3-kinase. *Atherosclerosis* 1999;142:47–56. [PubMed: 9920505]
48. Aoudjit L, Stanciu M, Li H, Lemay S, Takano T: p38 mitogen-activated protein kinase protects glomerular epithelial cells from complement-mediated cell injury. *American journal of physiology Renal physiology* 2003;285:F765–774. [PubMed: 12837681]
49. Tang H, Amara U, Tang D, Barnes MA, McDonald C, Nagy LE: Synergistic interaction between C5a and NOD2 signaling in the regulation of chemokine expression in RAW 264.7 macrophages. *Advances in bioscience and biotechnology* (Print) 2013;4:30–37. [PubMed: 24634797]
50. Mu H, Ohashi R, Yan S, Chai H, Yang H, Lin P, Yao Q, Chen C: Adipokine resistin promotes in vitro angiogenesis of human endothelial cells. *Cardiovascular research* 2006;70:146–157. [PubMed: 16515776]
51. Xu Y, Luan Y, Liu S, Sun J, Wang K, Cai J, Jiang W, Yang P, Wei F, Qu X: Kif4 regulates the expression of VEGFR1 through the PI3K/Akt signaling pathway in RAW264.7 monocytes/macrophages. *International journal of molecular medicine* 2017;39:1285–1290. [PubMed: 28350061]
52. Morgan BP, Dankert JR, Esser AF: Recovery of human neutrophils from complement attack: removal of the membrane attack complex by endocytosis and exocytosis. *Journal of immunology* (Baltimore, Md : 1950) 1987;138:246–253.
53. Bronner F: Adaptation and nutritional needs. *The American journal of clinical nutrition* 1997;65:1570. [PubMed: 9129494]

FIGURE 1.

Sub-lethal levels of complement activation using zymosan. A) Representative immunofluorescence image showing CK7 positive human cytotrophoblast cells in culture isolated from term placentas. B) Representative immunofluorescence image (200×) showing deposition of MAC complex on the surface of syncytiotrophoblast cells upon complement activation using 0.5% zymosan and 10% NHS. C) Quantification of immunofluorescence intensity of MAC on syncytiotrophoblast cells showing significantly higher deposition upon complement activation. D) ELISA quantification of MAC in the culture media of syncytiotrophoblast cells upon complement activation with different zymosan concentrations showing dose dependent release of MAC. E) LDH release assay showing negligible cytotoxicity on syncytiotrophoblast cells upon complement activation with 0.01, 0.1 and 0.5% zymosan. n=3–5, * $p=0.028$, ** $p=0.031$.

FIGURE 2.

Complement activation induced upregulation of sFLT1 in syncytialized human trophoblast cells. A) RT-qPCR of sFLT1 showing significantly elevated levels of sFLT1 mRNA in complement activated cells compared to no complement activation and complement activation using C3/C4 depleted NHS. B) Western blot showing increased levels of 3 isoforms of sFLT1 (145, 100 and 60 Kd) upon complement activation compared to NHS and zymosan negative control treatments. C) Representative immunofluorescence image (200x) showing increased immunostaining of FLT1 and its increased co-localization with CK7 immunostaining upon complement activation. D) Quantification of immunofluorescence intensity of FLT1 showing significantly higher expression of FLT1 upon complement activation. n=3-5, * $p=0.031$, ** $p=0.028$.

FIGURE 3.

Complement activation stimulated the release of sFLT1 depending on degree of activation.

A) Representative Western blot showing decreased levels of three sFLT1 isoforms with increasing degree of complement activation. B) The 100 Kd sFLT1 isoform band was quantified by density measurement. C) Levels of sFLT1 in cell culture media measured by ELISA. n=3–5. * $p=0.015$, ** $p=0.03$, *** $p=0.0003$, **** $p<0.0001$.

FIGURE 4.

Complement activation by antibody presensitization of syncytiotrophoblast cells induced upregulation and release of sFLT1 depending on the degree of activation. A) Representative image showing dose dependent binding of Anti-HLAG on syncytial trophoblast cells. B) ELISA quantification of MAC in the culture media of syncytiotrophoblast cells upon complement activation by antibody presensitization (Anti-HLAG for 1 hour) showing increased degree of activation with increased amount of antibody used for presensitization. C) RT-qPCR of sFLT1 showing significantly elevated levels of sFLT1 mRNA in antibody presensitized cells compared to NHS alone cells. D) Representative Western blot showing decreased levels of three sFLT1 isoforms with increasing degree of complement activation D) Levels of sFLT1 in cell culture media measured by ELISA. n=3–5, * $p=0.028$, ** $p=0.007$.

FIGURE 5.

Higher levels of MAC deposited on term placentas from preeclampsia compared to normotensive pregnancies. A) Representative images of MAC-C9 PLA in term villous tissues showing deposition of MAC on syncytiotrophoblast cells (green arrow, ST). Isotype controlled IgG were used in place of anti-C9 and anti MAC antibodies for negative control. B) Quantification of MAC by PLA on villous tissues showing significantly elevated levels of MAC deposition in preeclampsia (n=10) compared to normotensive (n=11) women. * $p=0.03$.

FIGURE 6.

Recombinant human C3a upregulated sFLT1 expression without inducing the release. A) RT-qPCR showing upregulation of sFLT1 mRNA in syncytial trophoblast cells treated with C3a (2 μ g/mL). B) Quantification of sFLT1 in syncytial trophoblast cells culture media showing that C3a did not induce the release of sFLT1. n=3–5, * $p=0.005$, ** $p<0.0001$.

FIGURE 7.

Correlation between sFLT1 and MAC released in to culture media. A) MAC levels in culture media positively correlated with sFLT1 levels. B) There was no correlation between cellular MAC levels and sFLT1 levels in culture media. $n=16$ reactions, Pearson $r=0.569$, $p=0.02$.