Published in final edited form as:

Placenta. 2008 October ; 29(10): 855–861. doi:10.1016/j.placenta.2008.07.008.

The C5b-9 membrane attack complex of complement activation localizes to villous trophoblast injury *in vivo* and modulates human trophoblast function *in vitro*

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Abstract

The complement system plays an important role in normal human pregnancy. Uncontrolled activation of this system has been associated with many disease states. We tested the hypothesis that the C5b-9 membrane attack complex (MAC) localizes to sites of villous injury and modulates trophoblast function. Placental sections from pregnancies with no complications, intrauterine growth restriction, or preeclampsia were immunostained and the surface density for MAC and fibrin was determined by morphometric analysis. Primary cytotrophoblasts from term placentas were cultured in a FiO₂ of < 1%, 8% and 20% with 10% human serum containing active MAC or heat-inactivated control serum. Immunofluorescent MAC binding to trophoblast was quantified, and the neoepitopes formed in cytokeratin 18 filaments and poly-ADP-ribose polymerase during apoptosis were used to measure cell death. Trophoblast differentiation was assessed by HCG secretion, formation of syncytia, and expression of syncytin.

MAC localized to fibrin deposits in normal placentas, and especially in placentas from IUGR and preeclampsia. MAC binding to cytotrophoblasts was inversely proportional to FiO₂ and enhanced apoptosis. MAC increased markers of differentiation in cultures at 72h (medium HCG, syncytia and syncytin expression). Our findings demonstrate that MAC associates with fibrin deposits at sites of villous injury *in vivo*. Hypoxia also enhances MAC deposition in cultured trophoblasts and MAC alters trophoblast function in a phenotype specific manner.

Introduction

Optimal placental function is a hallmark of normal pregnancy. Placental dysfunction associates with multiple maladies including preeclampsia and intrauterine growth restriction (IUGR). Insults such as hypoxia [1][2] and exposure to inflammatory cytokines [3] can injure placental villi. Previous studies from our laboratory [4] and others [5][6] have identified that sites of villous injury are marked by discontinuities in the syncytiotrophoblast layer where fibrin type

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fibrinoid is deposited on the trophoblast basement membrane. Importantly, these areas exhibit markers of apoptosis in the syncytiotrophoblast [7][8] and the fibrin matrix provides a scaffold for trophoblast to re-epithelialize the denuded villous surface [4]. Although evident in placentas from uncomplicated pregnancies, this injury and repair process is pronounced in pregnancies complicated by preeclampsia or intrauterine growth restriction [9]. How inciting agents such as hypoxia or re-perfusion induce trophoblast injury is poorly understood, but excessive injury commonly yields placental dysfunction and a pregnancy malady.

The complement cascade is activated through one of three routes; the classical, alternative or lectin pathways [10] [11]. All three pathways converge at the C3 convertase and share a common effector, the membrane attack complex (MAC). The MAC comprises a molecular complex of complement proteins, including C5b, 6, 7, 8, and 9. Excess binding of MAC to the surface of cells and bacteria enhances phagocytosis, induces inflammation and creates pore formation in surface membranes [10]. Importantly, sub-lytic activation of MAC activates a variety of signaling cascades in multiple cell types, including lymphocytes, endothelial cells, and glomerular epithelium[10]. Moreover, dysregulated complement activation in nonpregnant women mediates immunological injury in heart, lung, and kidney [12].

Unrestrained complement activation may play a role in the pathophysiology of several pregnancy disorders [13]. For example, the hypoperfused kidney of women with preeclampsia exhibits deposition of complement split products in glomeruli [14], mice null for the complement regulatory protein *cr1* show an embryonic lethal phenotype [15], and mice exposed to human anticardiolipin antibodies exhibit IUGR secondary to activation of the complement cascade [16]. Collectively, these data suggest that dysregulated activation of the complement cascade may contribute to sub-optimal pregnancy outcomes.

We questioned if complement deposition associates with villous injury in the human placenta and if the binding of MAC alters human trophoblast function. We tested the hypotheses that MAC co-localizes with fibrin type fibrinoid in normal and abnormal placentas *in vivo*. We then tested the hypotheses hypoxia enhances MAC deposition in cultured human trophoblasts and alters trophoblast apoptosis and differentiation.

Materials and Methods

Tissue procurement

This study was approved by the Institutional Review Board of Washington University School of Medicine. Placentas were obtained from singleton gestations with uncomplicated pregnancies (n=4), preeclampsia (n=4), or IUGR (n=4). Preeclampsia was defined by the criteria of the American College of Obstetrics and Gynecology [17]. IUGR was designated as birthweight < 10th percentile and histopathological examination of the placental pathology that was consistent with the diagnosis, as we previously described [18]. Four samples of villous tissues midway between maternal and fetal surfaces and midway between the periphery and center of the placental disc were sampled from each placenta. The specimens were immersion-fixed at room temperature in 10% neutral buffered formalin for 24 h, embedded in paraffin in random orientation, and sections of five micron thickness were processed for immunohistochemical staining.

Immunohistochemistry

Paraffin was removed from sections by a graded series of alcohols, and non-specific staining was blocked in 5% bovine serum albumin. Double immunofluorescent staining with antigen specific antibodies was used to identify fibrin (American Diagnostica Inc., Greenwich, CT; 1:100) concomitant with the neoepitope that develops on the C9 component of complement

when the MAC is formed (Quidel, San Diego, CA; 1:50). To avoid cross-reactivity, sections were initially incubated with anti-MAC for 1 h followed by donkey anti-mouse Alexa fluor 555 (Molecular Probes, Invitrogen, Eugene, OR). After washing, a second primary antibody, anti-fibrin IgG1 was directly labeled with an isotype specific, xenon excited Alexa-488 labeling kit following the instructions of the manufacture (Molecular Probes; 1:200). Specimens were washed 15 min in PBS prior to staining nuclei with TOPRO-3 iodide (Molecular Probes).

Morphometric analysis

Ten random fields per specimen were imaged for each control, IUGR, and preeclamptic placenta. Digital images were collected using the multi-channel mode for detection of argon and helium-neon laser emissions in a Nikon Eclipse E800 microscope equipped with a 20X objective and a C1 confocal scanning head. We used linear probes on a grid to overlay each digital micrograph, recorded the probe intersect of the trophoblast basement membrane on each villus and identified the presence or absence of fibrin, MAC, or fibrin-MAC co-deposits. The surface density was defined by the proportion of linear probes that intersected the villous surface and expressed the antigen(s) of interest relative to the total number of villous intersects [19].

Trophoblast isolation and culture

Cytotrophoblasts, isolated from placentas of 12 uncomplicated term pregnancies, were plated at 3.5×10^5 cells/cm², and cultured in DMEM for 24 h or 72 h as previously described [20]. Cultures in the 72 h paradigm were initially cultured in DMEM with 10% FBS in a FiO₂ of 20% for the first 48 h. Cultures were exposed for the 24 h preceding analysis to 20%, 8%, or < 1% oxygen with 5% CO₂ atmospheres in the presence of either 10% normal human serum with active complement or control medium with 10% human serum that was heated at 56 C for 30 min to inactivate complement. Medium was harvested and cultures were washed with PBS. Cells were fixed in 4% paraformaldehyde for 30 min or cell lysates were prepared for western analysis.

Immunocytochemistry

Cytotrophoblasts were cultured for 24 h in the presence or absence of active complement in < 1%, 8% and 20 % oxygen. Cells were washed with PBS and fixed with 4% paraformaldehyde. After blocking nonspecific reactivity with 5% bovine serum albumin, cells were incubated with anti-MAC for 1 h at room temperature, washed with PBS, incubated with donkey anti-mouse secondary antibody conjugated to Alexa fluor 488 (Molecular Probes), and counterstained for nuclei with TOPRO-3 iodide. To assess the binding of activated complement, ten random images were obtained from each paradigm and voxel intensity for each image was quantified. Moreover, confocal microscopy was utilized for Z-stack imaging and 3-D rendering to assess the location of activated MAC relative to the cell surface, identified by immunostaining trophoblasts permeabilized with methanol and exposed to goat polyclonal anti-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA 1:100) conjugated to donkey anti goat Alexa fluor 488 (Molecular Probes).

To assess morphological differentiation, cells cultured in each of the three oxygen tensions in the presence or absence of MAC for 24 h prior to harvest at 72 h, were exposed to anti-desmosomal antibody (Sigma, Saint Louis, MO; 1:100), surface membranes were identified by incubation with secondary antibody conjugated to Alexa fluor 488 (Molecular Probes) and nuclei were counterstained by TO-PRO-3 iodide. Forty random images at 20X magnification were quantified for the number of individual syncytiotrophoblasts, the number of nuclei within each multinucleated syncytiotrophoblasts, and the number of cytotrophoblasts. We defined syncytiotrophoblasts as cells with ≥ 2 nuclei within the same plasma membrane bound structure, as determined by desmosomal staining.

To assess if apoptosis in cultures at 72 h affected trophoblasts in a phenotype specific manner, we conducted the above assessment of multi- and mono-nucleated trophoblasts in cultures harvested at 72 h with or without 24 h of active MAC in < 1% oxygen. We simultaneously assessed them for a marker of nuclear apoptosis, using an antibody that identified the neoepitope that develops in the poly-ADP-ribose polymerase antibody (PARP; Promega, Madison, WI; 1:100) during the cell death process. Incubation with anti-PARP was overnight at room temperature and after washing with PBS, we visualized PARP staining with a donkey anti-goat Alexa fluor 488 as previously described. The number of PARP positive nuclei in syncytiotrophoblasts and cytotrophoblasts was expressed as a ratio of total nuclei identified by TO-PRO-3 iodide staining.

Western immunoblotting

Cells were lysed in radio-immunoprecipitation assay buffer with a protease inhibitor; lysates were sonicated on ice for 10 sec and centrifuged. Thirty micrograms of cell lysates were analyzed and quantified for protein expression as previously described [21]. Antibodies used were the M30 antibody (Roche Applied Science, Indianapolis, IN; 1:250) that detects the neoepitopes formed from cleavage of cytokeratin 18 intermediate filaments as a measure of apoptosis and anti-syncytin antibody as a measure of trophoblast morphological differentiation (Santa Cruz Biotechnology, Santa Cruz, CA; 1:250).

Beta-HCG assay

Medium harvested from 72 h cultures was analyzed for HCG by ELISA following the manufacturer's instructions (DRG International, Germany). Background from medium only controls was subtracted and hormone levels were normalized to cellular protein.

Statistical analysis

Significance was assigned at a $p < 0.05$ and determined only after correction for multiple comparisons. A chi-square analysis of results from placentas of control, uncomplicated pregnancies assigned fibrin as the independent variable and MAC as the dependent variable. A subsequent analysis assigned each of the three clinical conditions as the independent variable for chi-square analysis to compare the surface density for fibrin, MAC, and fibrin-MAC co-deposits between placental sections of uncomplicated pregnancies and sections from pregnancies complicated by preeclampsia or by IUGR.

In the cell culture studies, the statistical analysis for continuous variables was done by ANOVA with Bonferroni correction. This included comparisons of densitometry for cytokeratin 18 and syncytin expression and medium HCG levels. Nuclei in syncytia in the presence or absence of MAC, and PARP positive nuclei vs. total nuclei in syncytiotrophoblasts and cytotrophoblasts was by chi-square analysis.

Results

MAC deposition co-localizes to sites of villous injury

Histological specimens from placentas of uncomplicated pregnancies exhibited fibrin containing fibrinoid deposits as eosinophilic, hypocellular areas on the surface of villi, where discontinuities in the syncytiotrophoblast layer indicated villous injury (Figure 1 A), as we previously described [4]. Immunohistochemical staining in the specimens from uncomplicated pregnancies (Figure 1 B, D) showed a surface density for fibrin of 5.6 % and for MAC of 7.1% (Table 1). The surface density for fibrin-MAC co-localization was 4.8 %. Importantly, a significant ($p < 0.01$) association of fibrin, as an independent variable, and MAC, as the dependent variable, was apparent in placental sections from uncomplicated pregnancies (Table

1). Indeed, MAC immunolocalized to 85.5% (165) of the 193 fibrin containing deposits present on the surface of villi.

We next examined the surface density of MAC, fibrin and co-localized MAC-fibrin complexes in placental sections from control pregnancies without complications and compared each to pregnancies complicated by preeclampsia or IUGR (Fig. 1 C; Table 1). The level of fibrin deposits was more than two-fold higher ($p < 0.05$) in both preeclampsia and IUGR, compared to control pregnancies. Moreover, the surface density of MAC was significantly higher ($p < 0.05$) in preeclampsia and in IUGR, compared to control placental villi (Table 1). Importantly, the strong association ($p < 0.01$) of fibrin with MAC co-localization was again apparent on villi from pregnancies with either preeclampsia, (93.3%; 350/375) or IUGR (91.0%; 353/388).

Hypoxia enhances MAC deposition on primary trophoblasts in culture

The cytotrophoblast phenotype was dominant at 24 h while the syncytiotrophoblast phenotype was prominent at 72 h, as previously described [20]. Primary cultures were grown either 24 h or 72 h - in a FiO_2 of 20%, 8%, and $< 1\%$ in the presence of serum with active MAC for the 24 h prior to harvest. MAC was localized in both syncytiotrophoblast (Fig. 2A) and cytotrophoblast (not shown) phenotypes as punctate, discontinuous immunofluorescence on the surface of the cells, as visualized in 3-D renderings obtained after confocal microscopy. Control cultures treated with heat-inactivated serum exhibited negligible fluorescence in all three oxygen paradigms (not shown). Importantly, the level of MAC deposition was inversely proportional to oxygen concentration in primary trophoblasts cultured with serum containing active complement (Figure 2).

MAC modulates apoptosis in trophoblast

We questioned whether MAC binding to the cell surface altered apoptosis in cultured human trophoblasts. Primary human trophoblasts were exposed for 24 h prior to harvest to normal human serum or heat-inactivated, control serum. Interestingly, MAC exposure protected trophoblasts from apoptosis when cultures in a FiO_2 of 20% were harvested at 24 h (not shown) or 72 h (Figure 3), as assessed by western analysis for cytokeratin 18 expression. In contrast, MAC enhanced trophoblast cell death by the same measure at both 24 h (not shown) and 72 h in cultures exposed to either a FiO_2 of $< 1\%$ or 8% (Figure 3).

MAC enhances differentiation of human villous trophoblasts

We next assessed the influence of MAC on two key features of trophoblast differentiation, secretion of HCG and formation of syncytiotrophoblasts. Primary trophoblasts cultured in $< 1\%$, 8%, or 20% oxygen were exposed for 24 h prior to harvest at 72 h to serum with active MAC or control serum. Cultures in a FiO_2 of $< 1\%$ or 8% exposed to active MAC exhibited significantly ($p < 0.05$) higher HCG levels compared to cultures exposed to heat inactivated control serum (Figure 4 A).

Mononucleated cytotrophoblasts *in vitro* fuse to form syncytiotrophoblasts and thereby simulate the morphological differentiation that occurs on placental villi *in vivo*. We found that MAC exposed cultures significantly increased ($p < 0.05$) the number of nuclei in syncytiotrophoblasts under all oxygen tensions, compared to control cultures exposed to heat inactivated serum (Figure 4 B). To buttress our results, we analyzed expression of syncytin, a protein that participates in membrane fusion into syncytium. We found enhanced syncytin expression in cells exposed to MAC, compared to cells exposed to control serum (Figure 4 C). These data indicate that MAC exposure enhanced not only hormonal differentiation but also morphological differentiation of trophoblasts.

MAC affects trophoblast apoptosis and differentiation in a phenotype specific manner

We exposed cultures to serum with active MAC or control serum for 24 h prior to harvest at 72 h and counted apoptotic nuclei separately in cytotrophoblasts and syncytiotrophoblasts. We used multi-fluorescent probes as described in Methods to outline surface membrane desmosomes, nuclei in cytotrophoblasts and syncytiotrophoblasts, and nuclei in both phenotypes that expressed the neoepitope indicative of apoptosis in the enzyme poly-ADP-ribose polymerase (PARP). As illustrated in the confocal image of Figure 5, we found that apoptotic nuclei were predominantly and significantly ($p < 0.05$) localized to the cytotrophoblasts (26 PARP positive/263 total nuclei; 9.9%) with few apoptotic nuclei in syncytiotrophoblasts (3 PARP positive/143 total nuclei; 2.1%) in the 72 h cultures. Formation of syncytiotrophoblasts by 72 h was lower in the presence of $< 1\%$ oxygen, compared to the 20% oxygen of standard culture conditions, as we previously showed [20]. The above results are consistent with the premise that MAC increases apoptosis in the cytotrophoblast phenotype, with little or no effect on apoptosis in syncytiotrophoblasts.

Discussion

Our studies of placental villi are novel as they correlate the terminal complex of complement activation, MAC, with the histopathology of villous injury in not only uncomplicated pregnancies but especially in pregnancies with preeclampsia or IUGR. The data show that MAC co-localizes to areas of fibrin containing fibrinoid deposited on the trophoblast basement membrane at discontinuities in the syncytiotrophoblast. MAC binding to the surface membrane of cultured trophoblasts is inversely related to oxygen tension. This MAC binding increases apoptosis in cytotrophoblasts exposed to lower than ambient oxygen tensions. Importantly, exposure to MAC enhances hormonal and morphological differentiation of trophoblasts grown in hypoxic culture conditions. The data support the premise that local complement activation plays a role in villous injury and repair with a phenotype dependent biological response, inducing apoptosis in cytotrophoblasts and facilitating differentiation of syncytiotrophoblasts.

The villous trophoblast, like other plasma-exposed surfaces [22], is continuously subjected to low levels of complement activation. Levels of C3a, C4a, and C5a are significantly higher in pregnant women compared to non-pregnant controls [23]. Preeclampsia is associated with even higher plasma levels of C3a and C5a compared to normotensive controls, a reflection of dysregulated activation of the complement cascade [24]. Immunohistochemical studies of human placentas show that cleaved complement proteins localize to trophoblast and stroma of villi and basal plate in normal pregnancy [25] [26] [27]. Our study is the first to correlate MAC deposition with the presence of a characteristic histopathological lesion. MAC deposition on trophoblasts exposed *in vivo* to villous hypoxia may directly contribute to trophoblast injury by triggering apoptosis in cytotrophoblasts, as shown by our *in vitro* findings. The lectin pathway may be one source for enhanced complement activation. Higher than non-pregnant levels of mannose binding lectin are present in normal pregnancy [28] and ficolins involved in the lectin complement pathway are expressed at higher levels in the placentas of preeclamptic compared to normotensive pregnant women [29].

MAC co-localized with fibrin deposits especially in placentas from pregnancies with preeclampsia or IUGR. MAC deposition with fibrin deposits at sites of villous injury is common to both pregnancy maladies even though preeclampsia and IUGR have otherwise different placental features [9]. Preeclampsia and IUGR are both associated with underperfusion, hypoxia and oxidative stress [3] and these are stimuli for complement activation. Moreover, unexplained fetal death *in utero* is accompanied by higher than normal maternal levels of C5a [23], and women with circulating antiphospholipid antibodies exhibit higher than control levels of C3b and C4d deposited on trophoblast in placental sections [30].

Collectively, these findings suggest that dysregulated complement activation may be a common mechanism for placental injury, independent of the etiology of the clinical malady.

We identified an inverse relationship between oxygen tension and MAC deposition on human trophoblasts while also establishing that MAC alters trophoblast apoptosis and differentiation *in vitro*. Sub-lytic levels of MAC alter function in cardiac myocytes [31], oligodendroglia [32], and renal epithelium [33]. Our *in vitro* data show MAC binding yields a phenotype related response of villous trophoblasts. We used heat-inactivation to eliminate the MAC binding as a control and the process of heat-inactivation of serum may also alter growth factors or other proteins in the serum. What is clear is that the responses of the trophoblasts correlate with enhanced binding of MAC. The cytotrophoblast phenotype at 24 h exhibits enhanced apoptosis but the cultures with prominent syncytiotrophoblasts at 72 h show higher levels of HCG as a marker of biochemical differentiation and more nuclei in syncytia and higher expression of syncytin as complementary markers of morphological differentiation. This seemingly contradictory relationship, with enhanced apoptosis in cytotrophoblasts yet enhanced markers of differentiation in the cultures with prominent syncytiotrophoblasts, suggests that there are adequate cytotrophoblasts at 72 h to fuse into syncytium, despite the MAC induced loss by apoptosis of some of the cytotrophoblasts at this time. We therefore speculate that complement activation, whether from hypoxia, re-perfusion, or a related insult, may enhance MAC binding to trophoblasts and thereby mediate a part of the injury to the surface villous trophoblast layer. Concomitantly, sub-lytic MAC binding to trophoblasts, especially in the FiO_2 of 8% characteristic of villous tissues *in vivo* [34], may enhance formation and differentiation of syncytiotrophoblast and thereby facilitate repair of injured villous surfaces. Such repair would re-establish the integrity of the syncytiotrophoblast layer that regulates maternal-fetal exchange. Further studies are needed to test these speculations.

Acknowledgements

We would like to thank the National Institutes of Health for grant support from R01-HD29190 (DMN) and R01-HD45675 (YS).

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Figure 1.

A. Hematoxylin and eosin stained section of placenta from an uncomplicated pregnancy showing a villus, the intervillous space (IVS), and fetal vessels (FV). The discontinuity in the syncytiotrophoblast layer (arrows) where eosinophilic fibrin containing fibrinoid (fibrin) is deposited on the trophoblast basement membrane. B. Immunohistochemical staining of an area of injury in the trophoblast layer on a villus where fluorescence of fibrin staining in a fibrinoid deposit green co-localizes with red staining indicative of MAC. Arrows again indicate the discontinuity in the syncytiotrophoblast layer. C. Immunohistochemical staining for fibrin (green) and MAC (red) that was present in the placental sections from both IUGR (illustrated here) and preeclampsia (not shown). D. Control placental section incubated with non-immune serum.

Figure 2.

A. Using confocal microscopy, we obtained three-dimensional re-constructions of cells exposed to MAC, as described in Methods. Red fluorescence indicative of MAC appears as punctate, discontinuous deposits on the surface of trophoblasts, external to the plasmalemmal actin filament network marked by green fluorescence (upper left panel). MAC deposition was highest in the lowest oxygen tension in cultures of human trophoblasts exposed 24 h to normal human serum containing active MAC. B. Graphic quantified levels of MAC binding (y-axis) vs. oxygen tension (x-axis) for three experiments (* denotes $p < 0.05$)

Figure 3.

A. Western analysis for expression of the neoepitope in cytokeratin 18 intermediate filaments formed during apoptotic cell death of epithelium. A representative blot is shown. B. Quantified analysis of cytokeratin 18 expression from six experiments using different primary cultures analyzed by densitometry and normalized to actin expression as a loading control. Open bars represent control cells treated with heated serum and closed bars represent cells treated with normal human serum containing active MAC. *P < 0.05 compared to specimen at the same oxygen concentration

Figure 4.

A. Medium HCG levels (mean + SD) at 72 h for five primary cultures exposed to serum with active MAC (closed bars) or serum heated (open bars) to inactivate the complement cascade proteins. B. Immunofluorescent staining for desmosomes in green that marks surface membranes and TO-PRO 3 iodide staining of nuclei in blue, that allowed quantified analysis of the number of nuclei in syncytiotrophoblasts in cultures exposed to < 1% oxygen and serum with active MAC or heated control serum for 24 h prior to harvest. The Table shows the number of nuclei in syncytiotrophoblasts among 20 random fields in each paradigm from two separate primary cultures analyzed. Panel C shows the western analysis of syncytin in two primary cultures of cells exposed to 1% oxygen that were cultured in the presence or absence of active MAC. *P < 0.05

Figure 5.

Confocal microscopy was used to obtain a three dimensional reconstruction of primary trophoblasts cultured 24 h prior to harvest at 72 h in the presence or absence of MAC and stained for desmosomes to mark surface membranes (green) and for the neo-epitope that forms during apoptosis in the nuclear enzyme poly-ADP-ribose polymerase, or PARP, staining apoptotic nuclei green (white arrows). All nuclei in both phenotypes were stained blue with To-Pro-3 iodide. PARP positive cytotrophoblasts are labeled with short white arrows, non-apoptotic cytotrophoblast nuclei are marked with long white arrows, and non-apoptotic nuclei in syncytiotrophoblasts are labeled with a black arrow.

Table 1

Morphometric analysis of fibrin and MAC immunostaining in placental villi

Clinical Conditions: Total Intersects(TI):	Uncomplicated (n=4) 3454	Preeclampsia (n=4) 2615	IUGR (n=4) 2676
Surface Density:			
Without fibrin or MAC	3180/TI (90.0%)	2240/TI (85.6%)	2267/TI (84.75%)
Total fibrin	193/TI (5.6%)	375/TI (14.3%)*	388/TI (14.5%)*
Total MAC	246/TI (7.1%)	350/TI (13.4%)*	374/TI (14.0%)*
Fibrin and MAC	165/TI (4.8%)	350/TI (13.4%)*	353/TI (13.2%)*

*
p<0.01 compared to uncomplicated