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Half Year Progress Report

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Complement Activation and Preeclampsia

Specific aim 1: To identify the specific components of the activated C pathway involved in the synthesis and secretion of FLT-1 and sFLT-1 in syncytialized trophoblast cells isolated from human placenta

Experiments were performed to strengthen and fill some gaps in the preliminary data presented in this grant submission. The proposed experiments of specific aim 1 were completed as per the experiments proposed. The results are summarized below.

Primary trophoblast cells were obtained from placentas of five normotensive deliveries and used for examining the effect of complement activation on up-regulation and release of sFLT1.

Rsults:

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 cascade[18]. 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.

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 % zymozan, 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 ± 0.62ng/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) reveled 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 µg antibodies respectively, p=0.007).

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).

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 (2µg/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).

We published these results in “American Journal of Reproductive Immunology”

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We are in a good progress in performing the experiments proposed under specific aim 2.