Deciphering the role of C5a-C5aR axis in the sFlt1 production from endometrial stromal cells: Implications in preeclampsia

1. Project Summary

Preeclampsia is a pregnancy related hypertensive disorder. The symptoms of the disorder exhibit only after the 20th week of pregnancy. Untreated preeclampsia leads to eclampsia, a condition of multiple organ failure, coma or even death of mother. The pathophysiology leading to the disorder is currently unknown. Several theories including placental hypoxia, shallow invasion of trophoblasts and immunological pathways have been proposed for development of the disorder. sFlt1 (soluble form of VEGFR1) is the pathogenic molecule which is reported to be elevated during preeclampsia. Stromal cells of uterine endometrium (endometrial stromal cells; ESCs) also produce sFlt1. Decidual cells are differentiated ESCs by decidualization, a process which differentiates the more proliferative and less secretive stromal cells to less proliferative and more secretory decidual cells.

Complement system is important for our immunity. Among complements dissociated products of C3 and C5 namely C3a and C5a also act as anaphylatoxins. In particular C5a has an extensive role in activation of immune cells, pain responses in nerves, allergies etc. In preeclamptic patients, C5a deposition has been elevated in macrophages and C5aR (C5a Receptor) is upregulated in trophoblasts along with increase in the expression of sFlt1 in them. Yet the role of C5a-C5aR in sFlt1 expression remains to be studied in ESCs. This study aims at investigating the role of the C5a-C5aR axis in sFlt1 expression in ESCs. All the studies will be performed in human endometrial stromal cells (hESCs). The cells will be decidualized using excess cAMP. ESCs will be treated with C5a under both decidualized and non decidualized conditions. Initially sFlt1 expression will be quantified by real-time PCR, western blot and ELISA. This will be followed by studying the expressions of proteins including C5aR, transcription factors of sFlt1 gene like TFAP2A, CBP, PPAR-γ etc. Finally, c5aR will be knocked down in hESCs using siRNA followed by the treatment with C5a. Initially, the expression of sFLt1 will be checked followed by other proteins lists.

2. Objectives of the project

1. To determine the role of C5a-C5aR axis in sFlt1 production in normal and decidualized human endometrial stromal cell lines (hESCs).
2. To determine the status of C5aR when treated with retinoic acid and/in combination with c5a and check the sFlt1 production in normal and decidualized hESCs.
3. To check the effect of retinoic acid and C5a on C5aR knocked-down hESCs.

3. Detailed Methodology

3.1 Cell line and culture condition

Human Endometrial Stromal cell line (HESC) will be used for the studies. The cells will be grown in the ESC complete medium composed of DMEM/Hams F12 50/50 (Corning) supplemented with 10% FBS, and 1 % Penicillin/Streptomycin with 1% amphotericin B (Sigma). Cells will be maintained in T-Flasks in a humidified CO_2 incubator and the medium will be renewed every 4 days. For experiments cells will be seeded according to the recommended seeding densities and used for further studies.

3.2. Decidualization experiments

HESCs will be decidualized by treating the cells 0.5 mmol/L cAMP (N6,2′-O-Dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt; Sigma Chemicals) for 12 days in DMEM/High Glucose medium (Hyclone) with 5% charcoal stripped fetal bovine serum and 1 % Penicillin/Streptomycin with 1% amphotericin B (Sigma). The medium will be renewed every 4^{th} day.

3.3 Treatment with C5a, RA and BMS498

HESCs will be cultured as given in 3.1.1 and 3.1.2 followed by the treatment with C5a (optimal concentration), 0.1 μM RA and 1 μM BMS498 (Sigma). The cells will be treated for 96 hours followed by collection of cells and supernatant. Cells will be snap-frozen in liquid nitrogen and supernatant will be stored in -80^{o}C deep freezer until used for further experiments.

3.4 mRNA isolation

mRNA will be isolated from both the tissues and cells using Trizol. Trizol will be added to the tissues and cells immediately after harvesting and homogenized in a homogenizer. To the obtained sample 200 μL of chloroform will be added and incubated for 2-3 minutes. Then the sample will be centrifuged at 12,000 x g for 15 minutes. Three layers will be separated. The clear aqueous phase will be collected and 1 mL of isopropanol will be added to it. The solution will be incubated for further 10 minutes and the obtained pellet will be washed with 70% ethanol. Later the pellet will be allowed to air dry to remove traces of ethanol and the pellet will be dissolved in nuclease free water. This mRNA will be quantified and stored in -80°C for further use.

3.5 cDNA conversion

mRNA will be converted to cDNA using Superscript III reverse transcriptase (Invitrogen). Isolated mRNA will be added to random primers, dNTPs, 0.1 M DTT, 10x First strand buffer and RNase inhibitor and incubated in a thermocycler according to manufacturer’s instructions. Synthesized cDNA will be diluted before subjecting them to qPCR.

3.6 qPCR

SYBR green method will be used to determine the expression of various genes in both villous tissues and cells. The cDNA will be added to SYBR green master mix with primers of a specific gene and CoT values will be obtained. The 2ΔCt method will be used to compare the gene expression pattern between the experiments. Human ribosomal protein L17 will be used as a housekeeping gene.

3.7 Western Blot

The extracellular protein will be concentrated using heparin agarose and eluted in an extraction buffer. The intracellular protein will be isolated by sonication (with protease inhibitors) and will be used. The protein concentration will be determined. Briefly, SDS-Polyacrylamide gel will be prepared and proteins isolated from cells and prepared in Laemmli buffer will be loaded into the gel. The separated bands will be transferred to the nitrocellulose membrane and appropriate primary antibodies will be added. The solution will be incubated in rocker at 4^{o}C overnight followed by the addition of a secondary antibody. The developed bands will be studied in a Chemdoc. The proteins which will be studied but not limited to are C5aR, TFAP2A, ID1, RAR, PPAR-γ and CBP.

3.1.8 ELISA

The extracellular proteins will be studied by using ELISA (including prolactin and sFlt1). Briefly supernatant will be collected and diluted to an appropriate amount. Supernatant will be added to pre coated 96 well plate (R & D systems), followed by the addition of secondary antibody and substrate according to manufacturer's instructions.

3.1.9 C5aR siRNA knockdown

For knockdown experiments, the provided siRNA will be mixed with lipofectamine (Thermofisher) in a serum free medium and later added to the cells with the diluted final concentration of 10 nM. The expression of sFlt1 and other signaling molecules related with decidualization will be determined.

3.10. Fluorescent Microscopy

HESCs will be grown on coverslips. After treatment with RA, BMS498 and C5a for 96 h, cells will be fixed and permeabilized using 4% paraformaldehyde. The fluorescent labelled antibodies for C5aR will be added and kept in a rocker overnight at 4^{o}C. The resulting stained cells will be visualized under a fluorescent microscope and analyzed. DAPI will be used as a nuclear stain.

3.11. Flow cytometry

Flow cytometry work will be performed in the laboratory of Dr. M. Amudhan, Assistant Professor, Theni Government Medical College, Theni, TN, India. Immunophenotyping will be performed on the HESCs to identify the populations of interest using the following antibodies: C5aR, RARetc**.** All experiments in this study will be performed using a 13-color, 4-laser CytoFLEX S Flow Cytometer- Beckman Coulter.

4. Expected output:

The expected outputs are of two parts, one involving c5a and the other involving retinoic acid. We expect c5a to modulate the sFlt1 expression in both normal and decidualized cells. This will be confirmed using c5aR knocked down experiments. The molecular biology experiments will identify the intracellular signaling molecules which are involved in the signaling.