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Role of IL-10 in Preeclampsia

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***Abstract*: Preeclampsia has remained a symbolic and momentous public health threat in both developed and developing countries and according its contribution to maternal as well as prenatal morbidity and mortality worldwide. Unlike more prevalent cause of maternal mortality, medical interventions may be ineffective due to late presentation and appearance of cases. Throughout the world, the incidence of preeclampsia range between 2% and 10% of entire documented pregnancies. In this study, a border aspect of immune cells is highlighted to establish a relationship between these immune cells (like interlukin-10) and the case of preeclampsia.**

***Keywords*: Preeclampsia, Interlukin-10, IL-10.**

1. Introduction

Preeclampsia is a pregnancy-specific disorder characterized by hypertension and excess protein excretion in the urine. It is an important cause of maternal and fetal morbidity and mortality worldwide. The disease is exclusive to pregnant women and only delivery still continues to be the effective treatment. Preeclampsia is a disorder of widespread vascular endothelial malfunction and vasospasm that occurs after 20 weeks of gestation and can present as late as 4-6 weeks postpartum. It is clinically defined by hypertension and proteinuria, with or without pathologic edema. It is a multisystemic disorder and one of the most common hypertensive diseases of pregnancy that complicates 3%–8% of pregnancies in worldwide and constitutes a major source of morbidity and mortality. The incidence of preeclampsia ranges from 3% to 7% for nulliparas and 1% to 3% for multiparas women. Overall, 10%–15% of maternal deaths are directly associated with preeclampsia and eclampsia, (approximately 50,000 maternal deaths a year). Both hypertension and proteinuria implicate the endothelium as the target of the disease. The hypertension is characterized by peripheral vasoconstriction and decreased arterial compliance and proteinuria is associated with a pathognomonicrenal lesion known as glomerular endotheliosis, in which the endothelial cells of the glomerulus swell and endothelial fenestrations are lost. The abnormal placental development results in placental insufficiency and the releases excessive amounts of placental materials into the maternal circulation. This failure of formation of proper placenta leads to insufficiency of nutrition and oxygen (Hypoxia) to the growing fetus leading to intrauterine growth retardation and intrauterine deaths.

In this study we highlight the role of a specific cytokine known as IL-10 (INTERLUKINE-10) and its polymorphic effect on preeclampsia.

2. Status of preeclampsia worldwide

The incidence of preeclampsia, the precursor to eclampsia, varies greatly worldwide. WHO estimates the incidence of preeclampsia to be seven times higher in developing countries (2.8% of live births) than in developed counties (0.4%). Countries of North America and Europe is similar and estimated to be about 5-7 cases per 10,000 deliveries. On the other hand, incidence of preeclampsia in developing nations varies widely, ranging from one case for 100 pregnancies to 1 case for 1700 pregnancies. Rates from African countries such as South Africa, Egypt, Tanzania, and Ethiopia vary from 1.8% to 1.7%. In Nigeria, prevalence ranges between 2% to 16.7%

3. Pathophysiology of preeclampsia

An estimated 2-8% of pregnancies are complicated by preeclampsia, with associated maternofetal morbidity and mortality. In the fetus, preeclampsia can lead to ischemic encephalopathy, growth retardation with various other consequences. Eclampsia is estimated to occur in 1 in 200 cases of preeclampsia when magnesium prophylaxis is not administered. Preeclampsia is characterized by endothelial dysfunction in pregnant women. Therefore, the possibility exists that preeclampsia may be a contributor to future cardiovascular disease. In a meta-analysis study, several associations were observed between an increased risk of cardiovascular disease and a pregnancy complicated by preeclampsia. These associations included an approximately 4-fold increase in the risk of subsequent development of hypertension and an approximately 2-fold increase in the risk of ischemic heart disease, venous thromboembolism, and stroke. In a review of population-based study, Harskamp and Zeeman (2007) noted a relationship between preeclampsia and an increased risk of later chronic hypertension and cardiovascular morbidity/mortality, compared to women with normotensive pregnancy. Moreover, women who develop preeclampsia before 36 weeks' gestation or who have multiple hypertensive pregnancies were at highest risk.

4. Pathogenesis

The first decade of this millennium has witnessed major

advances in our understanding about the pathophysiology of preeclampsia. Historically known as the "disease of theories," the mystery about the molecular pathogenesis of preeclampsia is beginning to be unraveled with a key discovery about alterations in placental anti angiogenic factors. These anti angiogenic factors, such as sFlt1 and soluble endoglin, produce systemic endothelial dysfunction, resulting in hypertension, proteinuria, and the other systemic manifestations of preeclampsia. The molecular basis for placental deregulation of these pathogenic factors remains unknown, and the role of angiogenic proteins in early placental vascular development and trophoblast invasion is just beginning to be explored. Hypoxia is likely to be an important regulator. In addition, the renin-aldosterone-angiotensin II axis, excessive oxidative stress and syncytriotrophoblast debris, immune maladaptation, and genetic susceptibility may also all have roles in the pathogenesis of preeclampsia.

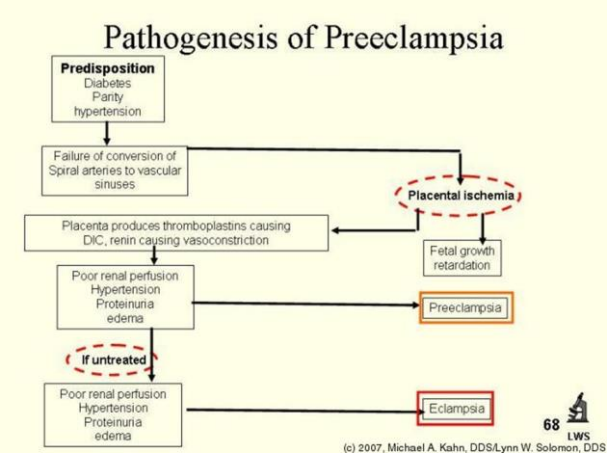

Fig. 1. Pathogenesis of preeclampsia

5. Genetics

Although most cases of preeclampsia occur in women without a family history, the presence of preeclampsia in a first-degree relative increases a woman's risk of severe preeclampsia two-fourfold. If a woman becomes pregnant by a man who has already fathered a preeclamptic pregnancy in a different woman, her risk of developing preeclampsia is almost doubled. Great Britain is a large study called The Genetics of Preeclampsia Collaborative (GOPEC) study that is collecting genomic information from 1,000 women with preeclampsia, along with the proband's parents, child, and partner to explore both maternal and fetal contributions to preeclampsia risk. From an evolutionary perspective, Flt1 variants may confer increased fetal fitness in sub-Saharan Africa from placental malaria and may be under natural selection in a malaria endemic area.

Other substances that have been proposed, but not proven, to contribute to preeclampsia include tumor necrosis factor, interleukins, various lipid molecules, and syncytial knots. Prothrombin, lipoprotein lipase, superoxide dismutase, nitric oxide synthetase, and Apo lipoprotein E these has been studied over a wide range.

6. Cytokines and IL-10

A. Cytokine

Cytokines are small secreted proteins released by cells have a specific effect on the interactions and communications between cells. Cytokine is a general name; other names include lymphokine, monokine, chemokine, and interleukin. Cytokines may act on the cells that secrete them (autocrine action), on nearby cells (paracrine action), or in some instances on distant cells (endocrine action).

B. IL-10

IL-10 is a type of cytokine belonging to the interleukin category and represents a family of its own. The IL-10 family includes IL-22 and represents a family of cytokines that inhibit inflammation and immune responses. IL-15 accomplishes nearly the same functions as does IL-2. Colony stimulating factors (CSF) such as IL-3, granulocyte-CSF, granulocyte-macrophage CSF, macrophage CSF, have overlapping functions but remain distinct gene products with specific receptor.

Interleukin-10(IL-10) was first reported by Mossman et al. under the name of cytokine synthesis inhibitory factor (CSIF) as a protein with the ability to inhibit the activity of inflammatory T-helper 1 (Th1)-type cells. Investigation led to the deduction that CSIF was the major factor that defined a difference between Th1- type and Th2-type T-cells as CSIF skewed T-cell activity toward inhibition. Although originally defined as a product of Th2 cells, this cytokine has now been shown to be produced by a wide set of cell types, including both immune and non-immune cells.

C. IL-10: A Pregnancy Compatible Cytokine

Several clinical observations regarding pregnancy implicate a role of an anti-inflammatory regulator such as IL-10. A significant number of women with rheumatoid arthritis (RA), an inflammation driven condition, consistently reported diminished symptoms during pregnancy. In contrast, women with systemic lupus erythematous (SLE), an antibody- driven autoimmune disease presented with increased symptoms during pregnancy. Taken together, these reports supported the postulate that an anti-inflammatory milieu, perhaps dominated by IL-10, was amplified during pregnancy most likely as a mechanism of tolerance toward the fetal allograft. Initial studies of the role of IL-10 during pregnancy were carried out in mice. Murine decidual tissues harvested across the spectrum of gestation showed that IL-10 was produced in supernatants and peaked at gestational day. In placental tissue obtained from normal pregnant women, immune histochemical analysis coupled with ELISA showed that IL-10 was produced in a gestational age dependent manner. Levels of IL-10 from first and second trimester placental tissues were significantly higher

than levels found in third trimester tissues, suggesting that IL-10 is intrinsically down regulated at term to prepare for the onset of labor programmed by production of an inflammatory milieu.

Further studies elucidated the crucial role of IL-10 at the maternal–fetal interface as placental and decidual tissue from first trimester missed abortions showed decreased IL-10 production when compared to control tissues obtained from first trimester elective terminations. Similarly, a comparison of placental tissue from elective caesarean (pre-labor) and placental tissue obtained post-labor showed higher IL-10 production in pre-labor tissues. Importantly, high IL-10 production in pre-labor tissues correlated to low prostaglandin-2 (PGE-2) levels, whereas the opposite held true for post-labor tissues. These data established IL-10 as a key contributor to the balance of pro-inflammatory versus anti-inflammatory signals that orchestrate proper pregnancy outcomes.

Investigation of pregnant IL-10 mice, when housed in a pathogen-free facility, showed that IL- 10 litter size and term of gestation were similar to those observed in congenic wild-type (WT) mice. Interestingly, IL-10 mice showed increased placental size and larger areas of maternal blood sinuses when compared to WT controls. These data elucidated a role of IL-10 as a mediator of placental growth and remodeling. It is noteworthy that extra villous trophoblast from first trimester exhibit poor IL-10 production while expressing high levels of message for matrix metalloproteinase-9 (MMP9), implying that invading trophoblast may temporally down regulate IL-10 expression to maintain their invasive, not necessarily endovascular, potential.

7. Cell Types Contributing To IL-10 Production at the Maternal–Fetal Interface

A. Trophoblasts

The maternal–fetal interface is composed of trophoblast cells of fetal origin intermingled with specialized maternal lymphocytes, stromal cells, and endothelial cells that comprise the decidua. The production of IL-10 by trophoblast cells and subsets of maternal uterine lymphocytes and summarize recent literature that delves into the intricate network of cellular cross talk that mediates this control. A conundrum in the field of reproductive immunology is the presence of uterine natural killer cells (uNK) throughout the decidua. NK cells operate through the missing self-hypothesis where lack of major histocompatibility complex (MHC) antigen presentation on a target cell leads to activation of the NK cells and resultant cytotoxicity.

In an organ that was once considered immune-privileged, it is difficult to rationalize the presence of NK cells. However, it has been postulated that the expression of non-classical MHC type I molecule HLA-G on trophoblast, particularly those with extra villous differentiation, plays a regulatory role in controlling NK cell cytotoxic activation. Interestingly, IL-10 has been shown to induce HLA-G on trophoblast. HLA-G is present in different isoforms and has become a focus of an intense debate for the exact role that it plays. While the mechanisms of HLA-G-based antigen presentation remain to be fully elucidated, the role of IL-10 as both a paracrine and autocrine regulator of trophoblast activity is apparent. Although villous cytotrophoblasts produce IL-10, it is not clear how trophoblast differentiation and invasion are controlled at this level. IL-10 decreases MMP9 transcription in villous cytotrophoblasts. This could be one mechanism by which cytotrophoblasts are selected for further differentiation and invasion. Compared to villous cytotrophoblasts, extra villous trophoblast is intrinsically poor in IL-10 production, thus allowing MMP expression and invasion competency. This may require paracrine activity within the placental meshwork. It is noteworthy that simultaneous activity of progesterone and IL-10 on trophoblast may work to sequester proinflammatory responses and to enhance regulated cross talk between the placenta and the decidua.

B. uNK Cells and Monocytes

Uterine NK cells and Monocytes are two major immune populations present in the decidua during pregnancy. These cells have the ability to produce and to be regulated by IL-10. Importantly, the characteristic immune functions of these cells, normally identified as cytotoxic killers, are altered in the context of pregnancy where their ability to aid in angiogenesis and placental regulation is paramount.

C. T Regulatory Cells

T regulatory (Tregs) cells in the decidua have recently come under the microscope of pregnancy research. Their characteristic ability to produce suppressive cytokines in response to foreign antigen makes Tregs promising therapeutic targets for intervention toward adverse pregnancy outcomes.

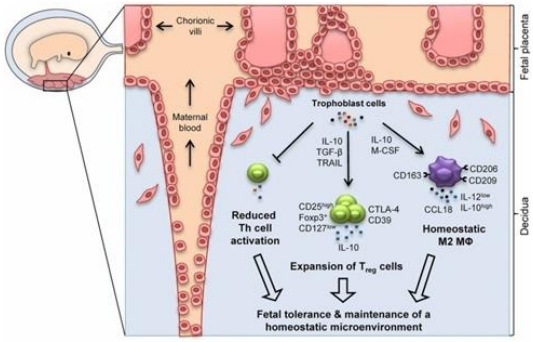

Fig. 2. IL10 By trophoblast cells

8. IL-10 Deficiency pregnancy and adverse pregnancy outcomes:

There have been several studies that couple IL-10 deficiency to adverse pregnancy outcomes such as recurrent spontaneous abortion (RSA), preterm birth, and preeclampsia. The mechanisms that may lead to poor IL-10 production at the maternal–fetal interface are not well understood. However,

polymorphisms in the IL-10 gene promoter have been associated with dysregulated IL-10 production and several diseases. Recent studies have identified five SNPs at -3575, -2849, -1082, -819, and -592 positions in the human IL-10 gene promoter. Similarly, the molecular effects of these SNPs in the IL-10 gene promoter remain to be elucidated in the context of pregnancy complications.

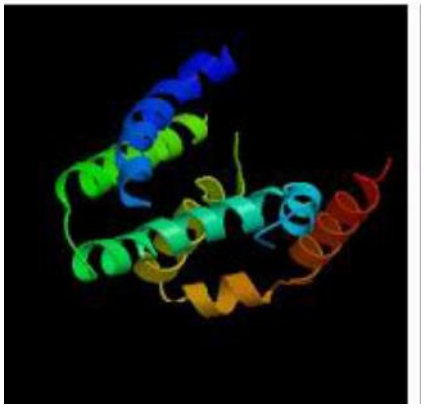

Fig. 3. This figure is an x-ray diffraction that shows il-10 as a monomer. Note that the native state of il-10, however, is homodimeric (pdb).

9. Functions

IL10 is small protein known as a cytokine that function as important regulator of immune system. Although IL-10 to have many different roles in the immune system. Its two major activities include inhibition of cytokine production by macrophages and inhibition of accessory functions of macrophages during T cell activation. The effects of this action cause IL 10 to play mainly as anti-inflammatory in immune system. IL10 was originally known as cytokine synthesis inhibitory factor and discovery of this protein is based on its biological activity. Recent reports provide evidence for genetically mediated regulation of il10 production. Although several polymorphic changes have been identified in the IL-10 gene promoter, three sites at the -1082(G/A), -819(C/T), and -592(C/A) positions have been best characterized for their regulatory influence.

Interleukin-10 is a multifunctional anti-inflammatory cytokine that is produced by various cells including monocytes, macrophages, B cells, T cells, and mast cells.IL-10, in return, modulates the performance of these various cells with important consequences for their ability to active and sustains immune and inflammatory responses. There is a large variation in IL-10 production capacity between healthy individuals. These inter individual differences in IL-10 production are largely under genetic control. In pregnancy, IL-10 is considered one of the major immune regulatory cytokines important for a successful outcome. A decreased with pregnancy loss and increase in preeclampsia. Role of IL-10 in relation to two aspects of human reproduction: the ability to have a child (fertility) and the probability of a couple conceiving in a specific period of time (fecundity). IL-10 levels have been shown to increase, decrease, or remain unchanged in women with preeclampsia. This study examines the difference in serum and placental IL-10 expression in women preeclampsia and investigates if the IL-10(-1082) A promoter polymorphism contributes to lower concentrations.

10. IL-10 Pathway

IL-10 (Interleukin-10) is a pleiotropic cytokine with important immunoregulator functions whose actions influence activities of many of the cell-types in the immune system. It is a cytokine with potent anti-inflammatory properties, repressing the expression of inflammatory cytokines such as TNF-Alpha (Tumor Necrosis Factor Alpha), IL-6 (Interleukin-6) and IL-1 (Interleukin-1) by activated macrophages. Functional IL-10R (IL-10 Receptor) complexes are tetramers consisting of two ligand-binding subunits (IL-10R-Alpha or IL-10R1) and two accessory signaling subunits (IL-10R-Beta or IL-10R2). Binding of IL-10 to the extracellular domain of IL-10R1 activates phosphorylation of the receptor-associated, JAK1 (Janus Kinase-1) and TYK2 (Tyrosine Kinase-2), which are constitutively associated with IL-10R1 and IL-10R2, respectively.

These kinases then phosphorylate specific tyrosine residues (Y446 and Y496) on the intracellular domain of the IL-10R1 chain. Once phosphorylated, these tyrosine residues and their flanking peptide sequences serve as temporary docking sites for the latent transcription factor STAT3 (Signal Transducer and Activator of Transcription-3). STAT3 binds to these sites via its SH2 (Src Homology-2) domain, and is, in turn, tyrosine-phosphorylated by the receptor-associated JAKs. It then homodimerizes and translocates to the nucleus where it binds with high affinity to SBE (STAT-Binding Elements) in the promoters of various IL-10-responsive genes. Constitutively active forms of STAT3 increase transcription of anti-apoptotic and cell-cycle-progression genes such as BCLXL, Cyclin-D1, Cyclin-D2, Cyclin-D3, and Cyclin-A, Pim1, c-Myc and p19 (INK4D).

IL-10 has also been reported to activate another major survival pathway consisting of PI3K (Phosphoinositide-3 Kinase) and its downstream substrates p70S6K (p70 S6-kinase) and Akt/PKB (Protein Kinase-B). Although the anti-inflammatory effects of IL-10 are not mediated via PI3K, the ability of IL-10 to promote survival of astrocytes or to induce proliferation of mast cells depends upon the activation of PI3K.

IL-10 also interferes with the activation potential of the p38/MAPK (Mitogen-Activated Protein Kinase) pathway, which is required to activate TNF (Tumor Necrosis Factor) translation. IL-10-mediated signals primarily target TNF-mRNA translation. This process is clearly dependent on TNF-3'ARE. IL-10 leads to ARE-dependent destabilization of granulocyte–macrophage colony-stimulating factor, granulocyte colony-stimulating factor and KC cytokine mRNAs. Inhibition of p38/MAPK affects TNF-mRNA translation by inhibiting LPS (Lipopolysaccharide)-induced

polysome coupling of TNF-mRNA in macrophages, without affecting TNF-mRNA accumulation. Similarly, the absence of MK2 leads to a profound reduction of LPS-induced TNF production, yet TNF-mRNA accumulation and stability are not affected. Thirdly, and most importantly, TTP (TNF-ARE Binding Protein Regulating-TNF Biosynthesis) exerts a potent destabilizing activity on TNF-mRNA without imposing translational control.

Two RNA binding proteins that have recently been demonstrated to have TNF-ARE binding capacities are TiaR and Tia1. The detailed mechanism by which IL-10 targets MAPK/SAPK (Stress-Activated Protein Kinase) signals is currently unknown. It is possible that IL-10 signaling interferes directly with the activation of MAPKs. There is evidence that points towards the requirement for JAK1/STAT3 in TNF suppression. Thus, one possibility would be that STAT3 activates factors capable of interfering with MAPK/SAPK signaling.

One of the IL-10-responsive genes, SOCS3 (Suppressor of Cytokine Signaling-3) is a member of a newly identified family of genes that inhibit JAK/STAT-dependent signaling. Moreover, the ability of IL-10 to induce de novo synthesis of SOCS3 in Monocytes correlates with its ability to inhibit expression of many genes in these cells, including endotoxin-inducible cytokines such as TNF-Alpha and IL-1. Both STAT3 and SOCS3 can be activated by inflammatory stimuli like LPS and TNF and, most importantly, SOCS3 activation can also be induced by STAT3-independent mechanisms. In addition, LPS can synergize with IL-10 to prolong SOCS3 mRNA stability in myeloid cells. Therefore, early post-transcriptional mechanisms may provide a pool of SOCS3, which can be rapidly activated in the absence of de novo gene transcription. Consequently, SOCS3 transcription may allow for the replenishment of a SOCS3 pool to enforce its negative action in a temporal manner, thus showing p38/MAPK dependency of both STAT3 and SOCS3 for their activation as well as for the apparent capacity of SOCS3 to suppress STAT3 activation itself.

IL-10 is a potent inducer of HO1 (Heme Oxygenase-1) in murine primary macrophages and J774 cell line. The induction of HO1 occurs at transcriptional level, and is mediated via a p38 MAP-kinase–dependent pathway. HO1 is involved in the biosynthesis of heme, and catalyzes a reaction producing CO (Carbon-monoxide), free iron, and the heme precursor biliverdin. Inhibition of HO1 protein synthesis by antisense oligonucleotide significantly reversed the inhibitory effect of IL-10 on the production of TNF-Alpha induced by LPS. HO1induction is also essential for the suppressive effect of IL-10 on LPS-induced expression of inducible nitric oxide synthase as well as matrix metalloproteinase-9. CO derived from heme degradation by HO1 mediates the anti-inflammatory effect of IL-10 in macrophages. IL-10 also promotes growth and survival of cancer cells, including non-Hodgkin's lymphoma, Burkitt lymphoma and non-small cell lung cancer. Recently, IL-10 has been demonstrated to directly increase survival of both cortical and cerebellar granule neurons, astrocytes and progenitor as well as differentiated oligodendrocytes.

11. IL-10 Gene polymorphisms:

The IL-10 gene comprise 5 exons, spans; 5.2 KB, and is located on chromosome 1 at q25.2 to q32.2. acc to Eskdel et. al; 1997. A number of groups have pursued intensive studies to identify naturally occurring gene polymorphisms in the IL-10 gene and flanking regions. To date at least 49 IL-10 associated polymorphisms have been reported, and an even larger number of polymorphisms are recorded in SNP databases [e.g., Ensemble Genome Browser]. Of these 49 polymorphisms 46 are SNPs, two are micro satellite polymorphisms, and 1 is a small (3bp) deletion. 28 polymorphisms occur in the promoter region of the gene, 20 polymorphisms are non-coding intronic or synonymous substitutions, and only one polymorphism results in a Change in amino acid sequence. Howell et al; 2001.The promoter polymorphisms have been subject to the most scrutiny, particularly with regarded to possible influences upon gene transcription and protein production. For example the IL-10 -1082SNP and -1082(G/A), -819(C/T),-592 haplotypes have been reported to be associated with differential IL-10 expression in vitro. the -1082 A,-819Ts,-592A haplotype is associated with decreased IL-10 expression when compared with the -1082G,-819C this is thought to reflect, at least in part, differential transcription factor binding associated with the -1082SNP.(Reuss et al;)Apart from that polymorphism in TNF-α (−308 G → A), IL-10 (−1082 G → A), IL-6 (−174 G → C), and IFN-γ (+874 A → T) are also suggested to be linked with preeclampsia .

A. IL-10 -1082 G/A Polymorphism in preeclampsia

The bi-allelic IL-10 promoter polymorphisms at positions −1082 G/A has been linked to high/low IL-10 production.

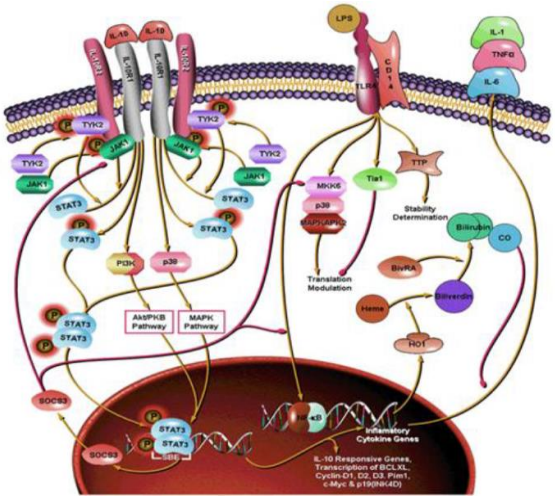

Fig. 4. IL-10 Pathway

12. Additional factors in preeclampsia

Various single nucleotide polymorphisms (SNPs) have been described in the IL-10 promoter region, both of distal and proximal promoter regions, with functional significance at positions other than -1082, like -819 and -592.

Other substances that have been proposed, but not proven, to contribute to preeclampsia include tumor necrosis factor, other interleukins like IL-1, IL-6, IL-12, various lipid molecules, and syncytial knots. sEng also plays a role in preeclampsia. SEng acts as a co-receptor for TGF-β, a potent proangiogenic molecule. sEng mRNA is upregulated in the preeclamptic placenta. Moreover, the extracellular region of sEng is proteolytically cleaved, and soluble sEng is released in excess quantities into the circulation of preeclamptic patients. In pregnant rats, sEng exacerbates the vascular damage that is mediated by sFlt1, resulting in severe preeclampsia-like illness, including the development of a HELLP-like syndrome and fetal growth restriction. In recent clinical studies, sEng was elevated not only during the disease but also before onset of symptoms. Elevations in sEng were particularly pronounced in women who developed preterm preeclampsia or preeclampsia with an infant who was small for gestational age. Although the gestational pattern of sEng concentration tended to parallel the trajectory of the sFlt1/PlGF ratio, multivariate analysis indicated that each was significantly associated with preeclampsia. Indeed, a composite measure that incorporated all three angiogenic molecules (sFlt1, sEng, and PlGF) was more strongly predictive of preeclampsia than the individual biomarkers.

13. Materials and methods

A. Sample collection

Sample must be collected from the appreciate choice of set of volunteers who are willing to involve and cooperate in the investigation. The sample collect must be adequate to produce results.

The clinical history of the patients revealed relevant pre eclamptic conditions associated with the repeated hypertension and proteinuria. 4ml of venous blood was collected in EDTA vacutainers from the patients and the controls and stored at -200C. The vacutainers with the blood are centrifuged and the plasma was separated and the remaining blood was used for DNA isolation. All the samples in the investigation were collected from Maternity Hospital Petla burj, Hyderabad.

B. Isolation of genomic dna using sucrose method:

The DNA was isolated from blood samples of all patients and control subjects by the sucrose method.

Chemicals preparation:

This method uses standard chemicals;

- *EDTA (0.5 M), PH 8.0:* Add 186.1 gr of anhydrous EDTA to 800 ml of distilled water. Adjust pH to 8.0 with NaOH pellets. Make up to 1 liter with distilled water. Autoclave at 15 p.s.i. for 15 min.
- *1 M Tris-HCL, P^H 7.6:* Dissolve 121.1 gr of Tris base in 800 ml of distilled water. Adjust pH with concentrated HCl. Allow mixture to cool to room temperature before finally correcting pH. Make up to 1 liter with distilled water. Autoclave at 15 p.s.i. for 15 min.
- *Preparation of red blood cell lysis buffer:* Add 10 ml of 1 M Tris, 109.54 gr of sucrose, 1.01 gr MgC12, adjust pH to 8.0 and finally add 10 ml of Triton X-100 to 800 ml of distilled water, and make up to 1 litter with distilled water. Autoclave at 15p.s.i.for 10 min. Sugars at high temperature can cause caramelization (browning), which degrades the sugars.
- *Preparation of nucleic lysis buffer:* Take 10 ml of 1 M Tris-HC1 (pH 7.6), 3.75 gr of anhydrous EDTA (pH 8.0), 10 gr SDS, 2.94 gr of sodium citrate, and adjust pH to 8.0. Make up to 1 liter with distilled water. Autoclave 15 minutes at 15 p.s.i.
- *Te buffer (ph 8.0):* Take 5 ml of 1 M Tris-HCl, pH 7.6, 2 mL of 0.5 M EDTA, pH 8, and make up to 1 liter with distilled water. Adjust pH to 8.0 and autoclave 15 min at 15. p.s.i.
- Chloroform prechilled TO 4°C.
- Ethanol (100%) prechilled TO -20°C.

C. Procedure of DNA extraction:

Before starting DNA extraction, liquid blood venogects should be shaken gently by rotating blood mixer (vortex)
500 µl of blood into a new 1.5 ml eppendorf tube and add 1000 µl of red cell lysis buffer.

1. Shake microfuge tube gently (up to homogenizing), then spin for 2 minutes at 7000 rpm
2. Discard supernatant and repeat steps 1-3 two or three more times to remove hemoglobin. It is important to breakdown the pellet by vortexing and rinses it well in red blood cell lysis buffer in order to clean the white blood cells from residual of hemoglobin.
3. Placing the tube on tissue paper for few seconds downward. Be careful from cross-contamination between different samples.
4. Add 400 µl of nucleic lysis buffer to eppendorf tube. Note: if the pellet formed, you must pipette the pellet up to dissolve it.
5. Add 100 µl of saturated NaCl (5M) and 600 µl of chloroform to eppendorf tube and mix on a rotating blood mixer at room temperature then spin it for 2 minutes at 7000 rpm.
6. Transfer 400 µl of supernatant to a new 1.5 ml tube.
7. Add 800 µl of cold (-20°C) absolute Ethanol and shake it gently then vortex it. DNA should appear as a mucus-like strand in the solution phase.
8. Spin the microfuge tube for one minute at 12000 rpm to precipitate, then discard supernatant carefully and let tube be completely dried in room temperature

(Place eppendorf tube downward on the tissue paper).

9. Add 50µl of TE to it then vortex; keep eppendorf tube of DNA in 4°C or -20°C for later uses. The DNA check of the sample is done using agarose gel electrophoresis. After completion of electrophoresis of the sample the gel is checked in gel documentation machine for the visualization of DNA as bands. Fig. 5 shows the gel picture of isolated DNA.
10. The isolated DNA is used about one µl per PCR reaction without adverse effects. DNA can be quantified and diluted to a working concentration at this point or simply use 1 µl per PCR reaction. It is expected that the yield of this procedure should be 100-300 ng/µl, DNA.
11.

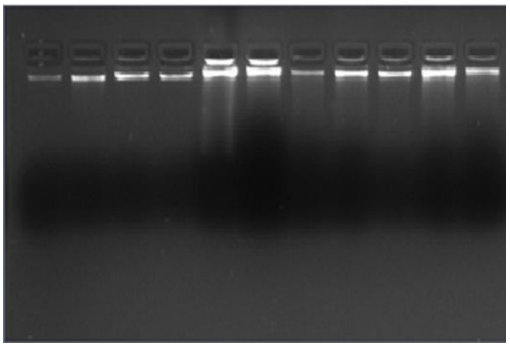

Fig. 5. Gel picture showing genomic DNA

D. Polymerase chain reaction

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase (after which the method is named) are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations.

1) Procedure

Typically, PCR consists of a series of 20-40 repeated temperature changes, called cycles, with each cycle commonly consisting of 2-3 discrete temperature steps, usually three. The cycling is often preceded by a single temperature step (called hold) at a high temperature (>90°C), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature(Tm) of the primers.

2) Initialization step

This step consists of heating the reaction to a temperature of 94–96 °C (or 98 °C if extremely thermo stable polymerases are used), which is held for 1–6 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR. (D.J. Sharkey)

3) Denaturation step

This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

4) Annealing step

The reaction temperature is lowered to 57 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically, the annealing temperature is about 3-5 degrees Celsius below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis. The melting temperature of a specific oligonucleotide primer (Tm) can be calculated by the following simple equation:

Tm= 2 (A+T) + 4 (G+C) - 5ºC

5) Extension/elongation step

The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–80 °C, (Chien A) (Lawyer FC) and commonly a temperature of 72 °C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.

6) Final elongation

This single step is occasionally performed at a temperature of 70–74 °C for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

7) Final hold

This step at 4–15 °C for an indefinite time may be employed for short-term storage of the reaction.

To check whether the PCR generated the anticipated DNA fragment (also sometimes referred to as the amplimer or amplicon), agarose gel electrophoresis is employed for size separation of the PCR products. The size (s) of PCR products is determined by comparison with a DNA ladder (a molecular

weight marker), which contains DNA fragments of known size, run on the gel alongside the PCR products (Conditions and programming of PCR are described in table-1).

1. Denaturing at 94–96 °C.
2. Annealing at ~65 °C
3. Elongation at 72 °C.

14. Agarose gel electrophoresis

Aim:

To separate the amplified DNA using agarose gel electrophoresis

Reagents:

- 1X TBE Buffer
- Agarose (2%)
- Ethidium bromide

Principle:

Electrophoresis is used to separate molecules by same property. In the case of DNA, we can separate the molecules based on their size. The phosphate molecules that make up the backbone of DNA molecules have a high negative charge, thus in agarose gel electrophoresis, the DNA is forced to move through a sieve of molecules made of agarose. The end result is that larger pieces of DNA move slower than small pieces of DNA. The gel is stained with Ethidium bromide and visualized in presence of UV rays, as Ethidium bromide intercalates in between DNA strands thus getting excited in the presence of UV rays. Equipment:

1. Balance
2. Conical flask
3. Microwave
4. Gel box with comb
5. Gel documentation unit
6. Gel electrophoretic unit
7. Micro pipette and tips

Procedure:

Step 1: Mixing Gel

1. On the scale, weighed 2 grams of agarose onto a piece of weighing paper.
2. Added agarose to conical flask.
3. Added 100mL of 1X TBE to conical flask.
4. Swirled vigorously to thoroughly mix agarose.
5. Slurry was by opaque.

Step 2: Melting Gel

1. Put agarose and 1x TBE slurry into microwave.
2. Heated the mixture for 160°C for 3 minutes.
3. Swirled until mixture is clear.
4. Added 2µl of EtBr and mix properly.

Step 3: Pouring the Gel

1. Placed gel tray into casting chamber.
2. Added casting comb(s) into the appropriate slot(s).
3. Poured agarose into gel tray to about 5-7mm.
4. Let sit for at least 30 min, until gel was cool to touch and was opaque in appearance.
5. Carefully removed combs by pulling them upwards firmly and smoothly in a continuous motion. The remaining depressions were the wells into which your samples will be loaded.
6. Once set, placed gel and tray into gel rig, with wells on the left (cathode) side. Then, filled gel rig with 1X TBE sufficient to cover the entire gel.

Step 4: Loading the Gel

1. Cut a piece of paraffin and placed it flat on the bench top.
2. Using a pipette, placed small dots of 6X loading dye (About 1-2uL) onto paraffin, in rows of 8, 1 dot for Each DNA or PCR sample that would be loaded on the gel. It was not necessary to be exact, and it was not necessary to change the tip.
3. Using a pipette took 3 µl of product and pipetted it onto its corresponding dye dot, then mixed the sample and dye by pipetting up and down. Then, pipetted up the dye/sample solution and released it into the proper well.
4. Put gel box cover into place (this step was essential for your gel to run and to minimize the risk of electric shock). Turned on power supply. Run the gel for 75 minutes at 80 volts.

Step 5: Visualizing the Gel

1. Turned off power supply. Using gloved hands, removed the cover from the gel box.
2. Removed the gel and casting tray from rig, avoiding dripping buffer all over the bench top.
3. Carefully placed the gel inside the gel documentation system.
4. Observed the gel documentation unit.

1) Analysis

After electrophoresis the gel was illuminated with gel documentation unit. The apparatus also contained imaging apparatus that takes an image of the gel.

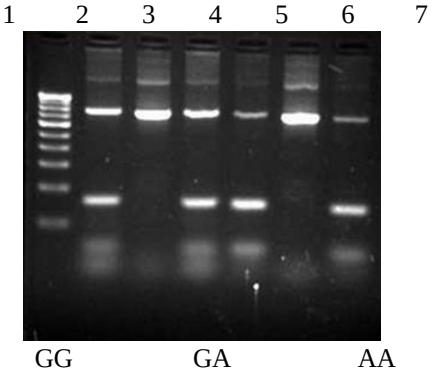

Fig. 6. Agarose gel

The figure shows a 2% agarose gel with the ARMS-PCR products for IL-10 (-1082) G/A polymorphism. The gel shows the Ladder (L): 100bp Size DNA Marker, HLA-Human leucocyte antigen; an internal control and the product band of 169bp. The Lane 1 shows the 100bp ladder; Lane 2 & 3 shows

Table 1
Demographic Characteristics of Control subjects

S. No.	Age	No. of Children	Consanguinity	Mode of Delivery	Blood Pressure
1.	28	1	NO	Normal	120/80
2.	21	2	NO	C-Section	120/80
3.	24	1	NO	Normal	110/80
4.	22	1	NO	Normal	100/70
5.	25	1	NO	C-Section	110/80
6.	26	1st pregnancy	NO	Normal	110/80
7.	20	3	NO	C-Section	110/80
8.	22	1st pregnancy	NO	C-Section	100/70
9.	20	1st pregnancy	NO	Normal	110/80
10.	20	1	NO	C-Section	120/80

Table 2
Demographic Characteristics of Preeclampsia Patients

S. No.	Age	No. of Children	Consanguinity	Mode Of Delivery	Blood Pressure
1	28	1	YES	C-Section	130/90
2	20	1st pregnancy	YES	C-Section	120/90
3	30	2	YES	C-Section	140/90
4	19	1st pregnancy	NO	C-Section	130/90
5	24	2	NO	C-Section	130/80
6	40	2	NO	C-Section	150/90
7	25	2	NO	C-Section	110/80
8	28	4th pregnancy(3 abortions)	NO	C-Section	140/90
9	20	1st pregnancy	YES	Normal	120/90
10	19	1st pregnancy	NO	C-Section	180/90

the homozygous GG; Lane 4 & 5 shows heterozygous GA and Lane 6 & 7 shows homozygous AA.

15. Results

Pre-eclampsia is a disorder resulting from poor placentation, although the pathogenesis of preeclampsia remains poorly understood, defective trophoblast invasion and spiral artery remodeling are thought to induce placental ischemia/hypoxia which eventually results in production of inflammatory molecules. Systemic presence of inflammatory molecules or dysregulation of essential proteins may then cause the maternal syndrome diagnosed by elevated blood pressure, proteinuria, kidney pathology, and oedema. Reduced production of IL-10 contributes to poor placentation and induction of inflammatory molecules.

In the control study 70% were below 25yrs and 30% were above 25yrs. 30% were in their first pregnancy, 50% in their second pregnancy, 10% in third and 10% in fourth pregnancy. None of them showed consanguinity. Of these controls 50% showed normal and 50% caesarean. All the controls subjects showed normal blood pressure.

In the present study 10 pre-eclamptic patients were considered out of which 80% were below 30yrs and 20% were above 30yrs. 40% of the cases were in their first pregnancy, 10% of them in second pregnancy and 40% of them were in third pregnancy. One of the cases showed previous history of 3 abortions. 40% of the cases had consanguineous marriages. Only 10% of the women showed normal delivery and remaining 90% showed caesarean. 10% of patients' have normal blood pressure remaining 90% of them showed high blood pressures (Table 2).

Table 3
Genotype Distribution and Allelic Frequencies of IL-10(-1082G/A) Polymorphism in Preeclampsia Patients and Controls.

Genotype	PE Cases		Controls	
	N	%	N	%
GG	3	30	5	50
GA	3	30	4	40
AA	4	40	1	10
Allele	N	Frequency	N	Frequency
G	9	0.45	14	0.7
A	11	0.55	5	0.3

A total of 10 preeclampsia cases and an equal number of controls were analyzed for the IL-10 (-1082 G/A) gene promoter polymorphism. Distribution of genotypes was GG 30%, GA 30% and AA 40% in PE cases and GG 50%, GA 40% and AA 10% in controls. The frequencies of G and A alleles were 0.45 and 0.55 in PE cases and 0.7 and 0.3 in controls respectively. The AA genotype and A allele was found to be increased in PE cases than in controls (Table 3)

16. Discussion

Pre-eclampsia is the most common pregnancy specific complication that still ranks as one of the major obstetric problems. It is a placenta-dependent pregnancy disorder. The syndrome is described as excessive maternal inflammatory response, directed against foreign fetal antigens that induce a chain of events including surface trophoblast invasion, defective spiral artery remodeling, placental infarction and release of pro-inflammatory cytokines and placental fragments in the systemic circulation. A normal pregnancy is accompanied by an inflammatory response which produces a state of mild systemic inflammation, completed with activation of multi-

components of inflammatory network. These inflammatory changes may progress to the point of circulatory de-compensation and endothelial dysfunction, resulting in one or more pregnancy complications, including preeclampsia.

In preeclampsia, an imbalance in the Th1/Th2 ratio is observed with deviation towards Th1 from Th2 response. Among the Th2 anti-inflammatory cytokines, IL-10 initiates and perpetuates inflammatory response. The IL-10 system may be involved in the regulation of Th1/Th2 cytokine production; since IL-10 can function as a co-stimulator for Th2 cell generation in both rodents and humans and may influence IFN-γ production mediated by natural killer (NK) and T cells. IL-10 is primarily an anti-inflammatory cytokine by its ability to stimulate the expression of genes associated with inflammation and immunity. Moreover, IL-10 has been implicated in implantation, trophoblast growth and invasion. Single nucleotide polymorphisms (SNPs) of the IL-10 gene were reported to be associated with susceptibility to inflammatory disease. Studies have shown that the levels of IL-10 synthesized both from decidual lymphocytes and peripheral blood mononuclear cells are higher in PE. The promoter region of the IL-10 gene contains a number of polymorphic sites including three single nucleotide polymorphisms (SNPs) at positions -1082, -819 and -592, which are related to high levels of IL-10 production.

The cytokine IL10 has pivotal roles in modulating immune and inflammatory processes . It is an important cytokine that regulates immune stimulation and inflammation via inhibition of cytokine synthesis by Th1 cells and macrophages and down regulates their expression. It is also a central regulator of the inflammatory response, acting to limit inflammation-induced tissue pathology by terminating monocytes and macrophage synthesis of TNF-α and an array of other proinflammatory cytokines and chemokines. Experiments in rodent models and humans implicates that, IL-10 can control inflammatory processes in pregnancy. Studies have shown that IL-10 is expressed abundantly in the decidual and placental tissues in humans as well as in mice. Elevated placental and amniotic IL10 synthesis occurs in pathologies of pregnancy, including intra uterine growth restriction and preeclampsia. Decidual T-lymphocytes from women with unexplained recurrent miscarriage are less frequently IL10 positive than clones from normal pregnancies.

Studies have shown that IL-10 may play an important role in maintaining pregnancy through corpus-luteum maturation, progesterone production and ensuring the establishment of Th2 cytokine environment. Furthermore, IL-10 promotes placentation and, together with transforming growth factor-β (TGF- β), is involved in the regulation of trophoblast invasion and proliferation. Polymorphisms in IL 10 genes can significantly influence the level of cytokine production.

17. Conclusion

The present study was undertaken to evaluate the role of IL-10 -1082 G/A promoter polymorphism in preeclampsia. The results showed increased frequency of AA genotype (low expresser) and A allele in preeclampsia cases when compared to control subjects. Our study is in concordance with the study by Vural et al. and Daher et al. where the presence of IL-10 AA genotype was associated with the development of preeclampsia. The work done by Makris et al. Demonstrated that even though the IL-10 genotype was AA, there was no significant lowering of IL-10 levels in maternal serum but when observed in placental tissue revealed a significant decrease in IL-10 concentration in placenta proving that IL-10 plays an important role in the placental formation. Low or inadequate expression of IL-10 results in disturbing the balance between pro-inflammatory and anti-inflammatory molecules and results in lowered anti-inflammatory action and may thus, contribute to the pathogenesis of preeclampsia. However, the result has to be confirmed in larger. Upon conclusion with larger studies, IL-10 can be used as a diagnostic tool in determination of Preeclampsia.

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