The Role of Regulatory T Cells in Preeclampsia

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Preeclampsia is a multisystem disorder of unknown cause that is unique to human pregnancy affecting about 7-10% of pregnant women. CD4+CD25\textsuperscript{bright} regulatory T (Treg) cells have been identified as a principle regulator of tolerance during pregnancy. The aim of this study was to demonstrate the change of peripheral CD4+CD25\textsuperscript{bright} regulatory T lymphocytes in normal pregnancy and preeclampsia, and to explore its role in the pathogenesis of preeclampsia. We determined CD4+CD25\textsuperscript{bright} Treg cells in the peripheral blood using flow cytometry and forkhead box P3 (FoxP3+) cells at the peripheral blood using real time PCR. 30 preeclamptic cases (group 1), 10 normal pregnancy subjects (group 2) and 10 non-pregnant healthy controls (group 3) were included. There was a highly significant decrease as regards Treg count and percentage in preeclamptic cases compared to normal pregnancy subjects ($P<0.001$), decrease in preeclamptic cases compared to non-pregnant healthy controls ($P<0.001$) and a significant increase in normal pregnancy subjects compared to non-pregnant healthy controls ($P<0.05$). There was a statistically significant decrease as regards RQ of foxp3 gene expression in preeclamptic cases compared to normal pregnancy subjects ($P<0.001$) and a significant increase in normal pregnancy subjects compared to non-pregnant healthy controls ($P<0.05$). There was no significant correlation between RQ and studied variables in preeclamptic cases ($P>0.05$). These findings suggest that the number of Treg cells are decreased in preeclampsia, and this decrease may break the maternal tolerance to the fetus.

Preeclampsia is a multisystem disorder of unknown cause that is unique to human pregnancy affecting about 7-10% of pregnant women. Research addressing this disorder has been extensive during the past decade, but has not resulted in substantial improvement in methods of prediction or prevention of the disorder. A major impediment in the development of such methods is our poor understanding of the various pathological mechanisms that lead to preeclampsia (Miko et al., 2009).

Maternal immune tolerance to paternal allo-antigens expressed by the fetus is a precondition of successful pregnancy in viviparous mammals. This occurs despite of exposure of the maternal immune system to potentially immunogenic fetal tissue. Local immune evasion mechanisms are thought to prevent maternal immune aggression towards the fetus (Aluvihare et al., 2004). The fetus constitutes a semi-allograft to the maternal host. To prevent fetal rejection, a system of tolerance must be present during pregnancy. Several explanations for such a maternal tolerance system have been proposed during normal pregnancy (Riley & Yokoyama, 2008).

Transplacental immune regulation refers to the concept that during pregnancy, significant cross-talk occurs between the maternal and fetal immune system with potential long-term effects for both the mother and child. In study done by Santner et al., (2013), they found a surprising observation that there is a strong correlation of peripheral blood regulatory T (Treg) cells between the mother and the fetus. In contrast, there is no significant Treg cell correlation between paternal fetal dyads (pairs), suggesting that the specific context of pregnancy, rather than the genetic parental similarity to the fetus, is responsible for this correlation (Santner et al., 2013). The changes in T cell subsets that may be seen in preeclampsia include low Treg activity, a shift toward Th1 responses, and the presence of...
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Th17 lymphocytes. B cells can participate in the pathophysiology of preeclampsia by producing autoantibodies against adrenoreceptors and autoantibodies that bind the AT1-R (angiotensin II type I receptor) (Laresgoiti-Servitje, 2013).

Regulatory T cells (Treg cells) represent a specialized subset of T cells with the capacity to suppress T cell responses involved in both autoimmune reactions and graft rejection (Steinborn et al., 2008).

Murine studies suggest that CD4+ CD25+ T cells provide mechanisms of specific immune tolerance to fetal alloantigens during pregnancy. In addition, a significantly higher percentage of CD4+ CD25+ T cells is present in decidual tissue compared with maternal peripheral blood. CD4+ CD25+ T cells are extensively studied by many groups for their regulatory capacities (Tilburgs et al., 2008).

It has been discovered that Tregs are themselves further composed of several subpopulations with specialized functions. Two major subsets of Tregs are termed natural Tregs (nTregs) and adaptive or inducible Tregs (iTregs). The nTregs are generated in the thymus and generally function to suppress immune responses to "self" antigens, while iTregs are generated outside of the thymus in various tissues and generally function to suppress immune responses to "foreign" antigens (antigens produced from proteins in cells foreign to your body). Researchers at Memorial Sloan Kettering Cancer Center in NYC demonstrated that it is iTregs and not nTregs that are critical to establish immune tolerance to the embryo and prevent miscarriage in mammals (Samstein et al., 2012).

In this case controlled study, we aim to investigate the changes in peripheral CD4+CD25+bright regulatory T lymphocytes number in pregnant women with preeclampsia to explore its role in the pathogenesis of the disease.

Subject and Methods

The study included 50 subjects from gynecology and obstetrics department and outpatient clinic in Benha university hospital. Study subjects were classified into 3 groups: Group (1): included 30 preeclamptic women at the age of 20-40 years old. They were diagnosed as blood pressure higher than 140/90 mmHg at 2 separate occasions, 6 hours apart, and significant proteinuria > 300mg /L in a 24 hour collection or a dipstick reading of >2+ on a voided random urine sample in absence of urinary tract infection. Any patient complicated by clinical chorioamnionitis or any infectious disorder was excluded from the study. Group (2): included 10 apparently healthy pregnant women who match the patient group for age. Group (3): included 10 apparently healthy non pregnant fertile women who match the patient group for age as control group. Informed consent was obtained from all subjects.

5 ml heparinized whole blood sample were obtained from subjects, and were divided as follows:

- Two ml were used to detect regulatory lymphocytes by flow cytometry with Becton Dickinson FACS calibur in Benha Children Hospital using Monoclonal Anti-human CD4 Fluorescein FITC and Monoclonal Anti-human IL-2 Ra (CD25)-Phycocerythrin (Becton Dickinson, San Jose, CA, USA).
- Three ml were used for extraction and detection of FOXP3 mRNA expression by Real Time PCR.
- Blood samples were processed within a 1-2 hours of collection.
- Total RNA were stored at −20°C in RNase-free water
- The concentration of RNA WAS determined by measuring the absorbance at 260 nm (A260) in a spectrophotometer. To ensure significance, A260 readings were greater than 0.15.

Flowcytometric Analysis: (Givan, 1992)

25 µL of washed cells were transferred to a 5 mL tube for staining with the monoclonal antibodies. PE (Phycocerythrin)-conjugated anti-CD25 and FITC-conjugated anti-CD4 reagents were added, incubated for 30 minutes at 8° C, washed twice in 4 mL of PBS. The cell pellet was resuspended in 200-400 µL of PBS for final flow cytometric analysis. Cells were immediately analyzed. Background fluorescence was determined using isotype-matched directly conjugated mouse anti IgG1/IgG2α monoclonal antibodies. Gating was placed around the lymphocytes based on their
forward scatter/side scatter profile, avoiding other cell populations. Cell populations were identified based on histograms and dot plots. The results were presented as percentage of cells stained with CD4 and CD25 antibodies.

Detection of FOXP3 mRNA by real time PCR: (Heid et al., 1996)

Extraction of RNA was done by QIAamp spin columns (supplied from Qiagen, Duesseldorf, Germany), according to the manufacturer’s instructions. The cDNA was prepared with High Capacity cDNA Reverse Transcription Kits (supplied from Applied Biosystems). Quantification of cDNA was done using the QuantiTect SYBR Green PCR kit (QIAGEN; Cat No. 204141; Lot No. 142317423) and corrected with GAPDH housekeeping control gene. FOXP3 primers (Integrated DNA Technologies, Coralville, IA) were as follows: forward primer, 5′-GCA CCT TCC CAA ATC CCA GT-3′; reverse primer, 5′-GGC CAC TTG CAG ACA CCA T-3′. A reaction mix was prepared as follows: 2x QuantiTect SYBR Green 10 µl, Primer A 0.5 µM, Primer B 0.5 µM, Template cDNA 1µg/reaction, RNase-free water to total reaction volume 25 µl. The Light Cycler was programmed as follows: PCR initial activation step 95°C for 15 minutes, denaturation step 94°C for 15 seconds, annealing step 60°C for 30 seconds, extension step 72°C for 30 seconds, and this was repeated for 50 cycles. To determine the level of FOXP3 expression, the differences (∆) between the threshold cycles (Ct) of the 2 genes were measured mathematically as follows: The relative Quantification (RQ) = 2 (ΔCt sample - ΔCt control).

Statistical Analysis

The program used was SPSS version 16. Quantitative data were analyzed using mean and standard deviation, while frequency and percentage were used with qualitative data. Student t test was used to compare means of different groups, while chi square to compare frequencies. Pearson correlation test was used to analyze relationship between two variables. A value of $P < 0.05$ were considered to indicate statistical significance.

Results

There was a highly statistically significant decrease as regards CD4+CD25$^{bright}$ cell count in preeclamptic cases (group 1) compared to normal pregnancy subjects (group 2) ($P<0.001$), and a highly statistically significant decrease as regards CD4+CD25$^{bright}$ cell count in preeclamptic cases (group 1) compared to non-pregnant healthy controls (group 3) ($P<0.001$). Also, there was a statistically significant increase as regards CD4+CD25$^{bright}$ cell count in group 2 compared to group 3 ($P<0.013$) (table1).

Table 1. Comparison of blood CD4+CD25$^{bright}$ cell count by flow cytometry between Preeclampsia and normal pregnancy:

<table>
<thead>
<tr>
<th>Variable</th>
<th>Groups</th>
<th>Mean± SD</th>
<th>Range</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+CD25$^{bright}$ cell count (cell/cmm)</td>
<td>Group(1)</td>
<td>70.21±33.7</td>
<td>22.6-163.8</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Group(2)</td>
<td>264.44±106.26</td>
<td>144.55-417.45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group(1)</td>
<td>70.21±33.7</td>
<td>22.6-163.8</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Group(3)</td>
<td>159.03±59.15</td>
<td>79.87-273.92</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group(2)</td>
<td>264.44±106.26</td>
<td>144.55-417.45</td>
<td>0.013</td>
</tr>
<tr>
<td></td>
<td>Group(3)</td>
<td>159.03±59.15</td>
<td>79.87-273.92</td>
<td></td>
</tr>
</tbody>
</table>

Group 1: preeclamptic cases
Group 2: normal pregnancy subjects
Group 3: non-pregnant healthy controls
$P<0.05$ is significant.
There was a significant decrease as regards Treg % in group 1 compared to group 2 ($P<0.001$), decrease in group 1 compared to group 3 ($P<0.001$) and a significant increase in group 2 compared to group 3 ($P<0.041$) (table 2).

There was no significant difference as regards absolute lymphocyte count between all studied groups ($P>0.05$) (table 3).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Groups</th>
<th>Mean± SD</th>
<th>Range</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treg % of total lymphocytes</td>
<td>Group(1)</td>
<td>2.82±0.89</td>
<td>1.2-5</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Group(2)</td>
<td>8.88±4.045</td>
<td>1.2-14.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group(3)</td>
<td>5.8±1.81</td>
<td>3.1-8.5</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variable</th>
<th>Groups</th>
<th>Mean± SD</th>
<th>Range</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute lymphocyte count/L</td>
<td>Group(1)</td>
<td>2446.0±746.74</td>
<td>1550-3600</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Group(2)</td>
<td>2664.0±621.08</td>
<td>1450-3450</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group(3)</td>
<td>2728.0±629.55</td>
<td>1560-3460</td>
<td></td>
</tr>
</tbody>
</table>

There was a highly statistically significant decrease as regards RQ of foxp3 gene expression in group 1 compared to group 2 ($P<0.001$) (table 4).

There was no significant correlation between RQ and studied variables in group 1 ($P>0.05$) (Table 5). There was a highly significant positive correlation between CD4+CD25$^{bright}$ cell count by flow cytometry and absolute lymphocyte count/L ($P<0.001$, $r=0.717$) (table 6).
Table 4. Comparison of relative quantity of foxp3 gene expression (RQ) between Preeclampsia and normal pregnancy using real time PCR:

<table>
<thead>
<tr>
<th>Variable</th>
<th>Groups</th>
<th>Mean± SD</th>
<th>Range</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RQ</td>
<td>Group (1)</td>
<td>0.611±0.36</td>
<td>0.067-1.65</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Group(2)</td>
<td>1.16±0.58</td>
<td>0.28-2.16</td>
<td></td>
</tr>
</tbody>
</table>

RQ: Relative Quantification
Group 1: preeclamptic cases
Group 2: normal pregnancy subjects
Sample gene expression = 2^\(-\Delta\Delta Ct\), RQ >1 = High expression, RQ 1 = Normal expression, RQ <1 = Low expression.

Table 5. Correlation between RQ and age, weeks of gestation, systolic blood pressure, diastolic blood pressure, absolute lymphocyte count/L and Treg % in preeclamptic case.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pearson correlation With RQ</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age(Y)</td>
<td>0.225</td>
<td>NS</td>
</tr>
<tr>
<td>Weeks of gestation</td>
<td>-0.092</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>0.022</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>0.062</td>
<td>NS</td>
</tr>
<tr>
<td>Absolute lymphocyte (count/L)</td>
<td>-0.124</td>
<td>NS</td>
</tr>
<tr>
<td>Treg %</td>
<td>0.146</td>
<td>NS</td>
</tr>
<tr>
<td>CD4+CD25^bright cell</td>
<td>0.013</td>
<td>NS</td>
</tr>
</tbody>
</table>

P>0.05 is not significant (NS).

Table 6. Correlation between CD4+CD25^bright cell count by flow cytometry and other clinical and laboratory variables in preeclamptic cases.

<table>
<thead>
<tr>
<th>Group(1)</th>
<th>Pearson correlation</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age(Y)</td>
<td>-0.25</td>
<td>NS</td>
</tr>
<tr>
<td>Weeks of gestation</td>
<td>0.037</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic Blood pressure (mmHg)</td>
<td>0.119</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic Blood pressure (mmHg)</td>
<td>0.197</td>
<td>NS</td>
</tr>
<tr>
<td>Absolute lymphocyte (count/L)</td>
<td>0.717</td>
<td>0.001</td>
</tr>
<tr>
<td>RQ</td>
<td>0.013</td>
<td>NS</td>
</tr>
</tbody>
</table>

P>0.05 is not significant (NS).
Example of a preeclampsia case
(decreased Treg cell population)

Example of a normal pregnancy

Example of a normal non pregnant woman

Figure 1. Dot plot showing Treg cells in preeclamptic case, normal pregnant woman and a non-pregnant one.
Figure 2. Fluorescence amplification plot of foxp3 gene mRNA expression in preeclamptic cases and normal pregnancy women.

Figure 3. Melting curve of foxp3 and GAPDH.

- Melting temperature of FOXP3: 86.33
- Melting temperature of GAPDH: 80.78
Discussion

Regulatory T cells (Tregs) are a specialized population of T lymphocytes known for their properties as potent suppressors of inflammatory immune responses and their ability to mediate immune homeostasis. Their unique properties, particularly their ability to suppress cytotoxically activated T cells and NK cells, make them an integral part of immune tolerance during pregnancy. These cells are characterized by the surface expression of CD4, CD25 and the intracellular forkhead box transcription factor, Foxp3 (Nevers et al., 2011).

The risk of preeclampsia may be increased by inadequate tolerance induction, data has shown that CD4+CD25+ Treg cells are essential in the maintenance of allograft pregnancy in mice (Aluvihare et al., 2004), and decreased levels of Treg cells are observed in the peripheral blood of spontaneous abortion patients (Sasaki et al., 2004).

In this study, the aim was to demonstrate the change of peripheral CD4+CD25^{bright} regulatory T lymphocytes in normal pregnancy and preeclampsia and to explore its role in the pathogenesis of preeclampsia. This study included three groups: group (1), which included 30 preeclamptic women at the age of 20-40 years old, group (2), which included 10 apparently healthy pregnant women who matched the patient group for age, and group (3), which included 10 apparently healthy non-pregnant fertile women who matched the patient group for age as control group.

In this study we should have done the FOP3 by gating on CD4+CD25^{bright} cells but this was not preferred since we were aiming at having a look at the regulatory T cell function using the Real time PCR.

In this study, There was a significant decrease as regards Treg count and % between in group 1 compared to group 2 (P<0.001), decrease in group 1 compared to group 3 (P<0.001) and a significant increase in group 2 compared to group 3 (P<0.05).

These findings are consistent with the study done by Somerset et al., (2004), who found that the percentage of CD4+CD25+ T cells was doubled during pregnancy (8.9%, range 3.4–28.1%) compared to controls (4.4%, range 2.3–8.0%; P < 0.0001).

Similar results were obtained from the study done by Sasaki et al., (2007) who found that population of CD25+CD4+ T cells in the Japanese and Polish pre-eclamptic cases (median 3.1%, range 1.9–7.9%) was significantly lower compared to that in the normal pregnant subjects (P < 0.0001) and in the non-pregnant subjects (P < 0.0001).

These findings also consistent with the study done by Steinborn et al., (2008) who found a significant decrease of the percentage of the CD4+CD25+ Treg subset in women with preeclampsia, (P<0.00001), but they found that in non-pregnant women, the percentage of CD4+CD25+Treg cells ranged from 3.1–9.1% (median 5.6%), and did not differ significantly from that of healthy pregnant women in the first trimester. The number of CD4+CD25+Treg cells, however, decreased significantly at the end of the first trimester and during the second trimester reaching minimum levels at mid gestation. Then, there was a significant increase until 30th week of gestation. From then on CD4+CD25+ Treg cell counts again decreased significantly until term. This may refer to the importance of the gestational age associated physiological fluctuations of CD4+CD25+Treg cells count and percentage.

Our research results were also consistent with those of the study done by Miko et al., (2009). They found that the percentage of CD4+CD25+T cells of gated lymphocytes was significantly lower in pregnant women with preeclampsia (0.42 vs. 0.82, P < 0.007)
than in non-pregnant controls, but the difference from healthy pregnant women did not reach the level of statistical significance (0.42 vs. 0.85). This may be explained by the difference in number of pregnant females included in our study (10) while it was (19) in their study.

But our study results were not consistent with the study done by Hu et al., (2008) who stated that the amount and the proportion of regulatory T cells were not significantly different among non-pregnant, normal pregnancy and pre-eclampsia groups (p>0.05 for both). There were no significant differences in the proportion and the amount of CD4+CD25+ regulatory T cells between mild and severe pre-eclampsia subgroups (P>0.05 for both). Also another study done by Paeschke et al., (2005) were not consistent with our results, as they did not find any significant differences in the levels of CD4+CD25+ Treg cells during preeclampsia and normal pregnancy in peripheral blood.

In this study, there was a highly statistically significant decrease as regards RQ of foxp3 gene expression in group 1 compared to group 2 (P<0.001).

According to Sasaki et al., (2007), the majority of CD4+CD25 bright T cells in pre-eclamptic cases, normal pregnancy subjects and non-pregnant women are FoxP3+ Treg cells. This indicates the importance of detection of FoxP3 marker in the setting of normal and complicated pregnancy, as an indicator of Treg cell activation. They found that the percentage of FoxP3+ cells within CD3+ T cells in the placental bed biopsy samples of pre-eclamptic cases were decreased compared to those in normal pregnancy subjects. These findings suggest that a decreased number of Treg cells was present in pre-eclampsia, and these changes might break the maternal tolerance to the fetus (Sasaki et al., 2007).

These findings also consistent with the study done by Prins, et al., (2009), who found that the frequency of FOXP3 + T cells in peripheral blood was significantly lower in women with preeclampsia than in healthy pregnant controls (P < 0.01).

The previous findings also consistent with the study done by Santner-Nanan et al., (2009) who found that CD4+ Foxp3+ cells were significantly lower in patients with preeclampsia (4.42%) compared with healthy pregnant women (6.26%) and nonpregnant controls (5.56%).

In 2008, Steinborn and his colleagues stated that there was a significantly decrease of the percentage of the FoxP3+Treg subset in women with preeclampsia than normal pregnant females (P<0.0001), but they found in non-pregnant women that the percentage of Foxp3+Treg cells ranged from 3.1–9.1% (median 5.6%), and did not differ significantly from that of healthy pregnant women in the first trimester. The number of Foxp3+Treg cells, however, decreased significantly at the end of the first and during and second trimester reaching minimum levels at mid gestation. Then, there was a significant increase until 30th week of gestation. From then on Foxp3+Treg cell counts again decreased significantly until term. Again this points to the importance of putting in mind the gestational age when interpreting the number of Foxp3+Treg cells in the normal pregnancy, and to the necessity of setting a reference interval for different stages of normal pregnancy (Steinborn et al., 2008).

During pregnancy there is an increase in the population of Treg cells which plays a role in maternal immune tolerance of her allogeneic conceptus. (Aluvihare et al., 2004). Our results support the hypothesis that Treg cells play a role in modifying the maternal immune response to the fetoplacental ‘allograft’ within the uterus.
Also, our results support other researches that point to maladaptation of the maternal tolerance in pre-eclamptic cases, as we found that the peripheral blood CD4+ CD25+ Treg cells were decreased in pre-eclamptic cases. The decreased CD4+ CD25+ Treg cells might augment the systemic inflammation in preeclampsia. These maternal immunological changes might reverse maternal tolerance, resulting in fetal rejection. It should be clarified whether reduced Treg cells are the cause or result in preeclampsia. Further studies are needed to clarify these points.

We recommend further studies on a wider scale for further evaluation of regulatory T cell count in each trimester of normal pregnancy, and to evaluate regulatory T cell count in different degrees of preeclampsia to confirm the relation between regulatory T cell and pathogenesis of preeclampsia.

References

