small to affect the results of the methylation analyses. In addition, sequence analysis did not show any mutations in *CDKN1C* (data not shown). These findings indicated that H19-DMR was aberrantly hypermethylated in both BWS patients and their associated placentas, but the aberrant methylation was consistently lower in the placenta, and that the H19-DMR GOM was strictly an isolated epimutation.

Finally, we analyzed the methylation status of 16 primary imprinted DMRs scattered throughout the genome-using COBRA (Fig. 2D and E). Only H19-DMR showed aberrant methylation among all primary DMRs in all samples, except for NNAT DMR, which was abnormal only in the placental chorangioma, indicating that the *IGF2/H19* imprinted domain was targeted for aberrant methylation in both somatic tissues and the placenta.

DISCUSSION

Methylation associated with parental imprints are erased in PGC and reestablished during gametogenesis in a sex-specific manner. The paternal pronucleus in the zygote undergoes active demethylation; extensive passive demethylation then ensues on maternal and paternal chromosomes during the pre-implantation period. After implantation, de novo methylation results in a rapid increase in DNA methylation in the inner cell mass (ICM), which gives rise to the entire embryo; in contrast, de novo methylation is either inhibited or not maintained in the trophoblast, which gives rise to the placenta [Li, 2002; Sasaki and Matsui, 2008]. The imprinted DMRs, however, escape these demethylation and de novo methylation events that occur in early embryogenesis. H19-DMR GOM in BWS patients is considered an error in imprint erasure in female PGCs [Horsthemke, 2010]. However, H19-DMR GOM, whether with or without microdeletions within H19-DMR, was partial, indicating a mosaic of normal cells and aberrantly methylated cells [Sparago et al., 2007; Cerrato et al., 2008]. These findings demonstrated that aberrant hypermethylation at H19-DMR was acquired after fertilization, although the precise timing was unknown.

Both participants in this study had isolated GOM at H19-DMR. The partial and variable hypermethylation among samples suggested epigenetic mosaicism. Furthermore, methylation levels in the placentas were lower than those in the blood and skin, suggesting that the aberrant methylation was acquired after implantation—when genome-wide de novo methylation normally occurs. Aberrant de novo methylation at H19-DMR is expected to be more widespread in the embryo than in the placenta, as this is normally the case for de novo methylation [Li, 2002; Sasaki and Matsui, 2008]; this disparity in efficiency could lead to the discordance between hypermethylation in trophoblast-derived placenta and that in embryo-derived blood and skin. This hypothesis is supported by a mouse experiment in which a mutant maternal allele harboring a deletion of four CTCF binding sites was hypomethylated in oocytes and blastocysts, yet was highly methylated after implantation [Engel et al., 2006]. To our knowledge, this is the first evidence demonstrating that aberrant hypermethylation of maternal H19-DMR is acquired after implantation in humans.

We found that of 16 primary imprinted DMRs analyzed, only H19-DMR showed aberrant methylation; even methylation at IG-DMR CG4, another paternally methylated, primary imprinted DMR, was normal in our patients. Although we only studied two patients, this finding indicated that the *IGF2/H19* imprinted domain in both the embryo and placenta was more susceptible than other imprinted domains to aberrant methylation acquired after implantation.

In conclusion, we found that methylation of H19-DMR was discordant in embryo-derived somatic tissue and placenta, strongly suggesting that the aberrant de novo methylation occurred after implantation. However, the precise mechanism of isolated H19-DMR GOM is still unknown. Since no mutations in *CTCF*, an important trans-acting imprinting factor, were found in these patients with isolated GOM at H19-DMR, the potential for mutations in the OCT and SOX transcription factors should be investigated because mutations of OCT-binding sites have previously been found in a few patients with H19-DMR GOM [Cerrato et al., 2008; Demars et al., 2010].

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Annexin A2 in amniotic fluid: Correlation with histological chorioamnionitis, preterm premature rupture of membranes, and subsequent preterm delivery

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Abstract

Aim: The aim of this study was to determine whether amniotic fluid levels of annexin A2, a phospholipid-binding protein that is abundant in amnion and regulates fibrin homeostasis, are associated with histological chorioamnionitis, preterm premature rupture of the membranes, and subsequent preterm delivery.

Materials and Methods: Amniotic fluid was obtained from 55 pregnant women with preterm labor and/or preterm premature rupture of the membranes before 32 weeks of gestation, and amniotic fluid levels of annexin A2 were measured with a sandwich enzyme-linked immunosorbent assay.

Results: Amniotic fluid levels of annexin A2 in patients with histological chorioamnionitis was higher than that in the remainder ($P = 0.053$), whereas amniotic fluid levels of annexin A2 in patients with preterm premature rupture of the membranes was significantly higher than that in the remainder ($P = 0.002$). Amniotic levels of annexin A2 was a fair test (area under receiver–operator characteristic curve = 0.679), and amniotic fluid levels of annexin A2 > 878.2 ng/mL had a sensitivity of 68.8%, a specificity of 65.2%, a positive predictive value of 73.3%, and a negative predictive value of 60.0% for predicting delivery within 2 weeks after amniotic fluid sampling. Furthermore, the combined use of amniotic fluid cut-off levels of 878.2 ng/mL for annexin A2 and 13.3 ng/mL for interleukin-8 improved the specificity (91.3%) and the positive predictive value (89.5%).

Conclusions: We identified amniotic fluid levels of annexin A2, especially in combination with amniotic fluid levels of interleukin-8, as a novel predictive marker for preterm delivery.

Key words: amniotic fluid, annexin A2, histological chorioamnionitis, interleukin-8, preterm premature rupture of the membranes.

Introduction

About 40% of all infant deaths in the USA are preterm-related.[1] Preterm births account for more than 50% of long-term morbidity. Intrauterine infection has not only been recognized as a major cause of preterm labor and delivery,[2] but has also been shown to be associated with an increased risk of neurodevelopmental impairments and respiratory and gastrointestinal complications in neonates.[3,4] Because intrauterine infection is confirmed through placental pathology as histological chorioamnionitis (hCAM), early diagnosis of hCAM

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clarifies the progress of infection and plays an important role in the management of patients.[5]

Microbiological cultivation is the classic 'golden standard' method for the determination of intrauterine pathogens; anaerobic bacteria, wall-less mollicutes (genus *Mycoplasma* and *Ureaplasma*), and viruses. However, the individual detection methods take time, are costly, and require experience. Recent advances in molecular biology and mass spectrometric detection enabled the rapid, highly sensitive, and specific global identification of both pathogens and host defense pathways of intrauterine infection or hCAM. The clinical biomarkers of intrauterine infections have been identified and include glucose, insulin-like growth factor-binding protein-1, cytokines, and matrix metalloproteinase (MMP) in amniotic fluid (AF).[6–9] The AF level of interleukin-8 (AF-IL-8) is an important index of hCAM and is a highly predictive marker for detecting preterm delivery before 34 weeks of gestation.[8]

The annexins are a family of calcium- and membrane-binding proteins expressed in most eukaryotic cell types.[10] Annexin A2 (AnxA2) has been implicated in membrane attachment, endocytosis, and exocytosis, which are pivotal activities for various pathogens to interact with host cells.[11–14] Furthermore, AnxA2 also functions as a modulator in immunologic clearance and for procoagulant and anticoagulant remodeling factors in vascular damage: phagocytosis,[15] von Willebrand factor secretion after histamine stimulation,[16] and fibrin clot dissolution.[17,18] It has recently become clear that certain types of dysregulation in annexin expression and activity can be correlated with human disease (e.g. the overexpression of AnxA2 in a patient with hemorrhagic form of acute promyelocytic leukemia[19] and the under-expression of annexin A5 on placental trophoblasts in the antiphospholipid syndrome[20]), which has led to the introduction of the term 'annexinopathies'.[21]

We previously identified that AnxA2 was one of the major targets of cord blood immunoglobulin M (IgM) in preterm infants with hCAM. The cord blood anti-AnxA2 IgM titer in preterm infants was correlated with the severity of hCAM.[22] This was the first report that the autoimmune response occurred in the fetal period – the fetal immune response syndrome. Because AnxA2 was expressed abundantly in amniotic epithelial cells, chorionic trophoblasts, villous trophoblasts, and villous endothelial cells,[23,24] we hypothesized that the fetal autoimmune response against AnxA2 might be triggered by the exposure of AnxA2 through inflammation and disruption around the membranes in hCAM. In this report, we constructed the sandwich enzyme-linked immunosorbent assay (ELISA) system using recombinant AnxA2[24] to measure amniotic fluid level of AnxA2 (AF-AnxA2). AF-AnxA2 seems to be correlated with hCAM and preterm premature rupture of the membranes (pPROM). The subsequent preterm delivery can be predicted by the combined assessments of AF-AnxA2 and AF-IL-8.

Materials and Methods

Amniotic fluid samples

After Ethics Committee approval was obtained from Toyama University, we collected AF specimens and baseline and outcome data. We studied 55 patients diagnosed with spontaneous preterm labor and/or pPROM before 32 weeks of gestation, who were managed at the Department of Obstetrics and Gynecology, Toyama University between January 2000 and November 2005. Preterm labor was defined according to the Canadian Preterm Labor Investigators group[25] as the presence of regular uterine contractions (six per 60 min documented by external tocography) or any uterine activity associated with a cervix effaced by at least 50% or dilated by 2 cm or more. To treat preterm labor, women with regular uterine contractions as defined above were treated with an intravenous infusion of the β-2 stimulant ritodrine hydrochloride (33 μg/min). When administration of ritodrine hydrochloride at the maximum dose (100 μg/min) was not effective, intravenous administration of magnesium sulfate was added (4 g/30 min), and then continued at 1–2 g/h.

After written informed consent was obtained, amniocentesis was performed in 55 cases. Amniocentesis was usually performed within a week of admission. The amniotic fluid samples were stored at −80°C until assays of ANXA2 concentrations. CAM was confirmed histologically in placental samples.

ELISA for detection of AF-AnxA2 and AF-IL-8

AF-AnxA2 was measured using a sandwich ELISA. Mouse anti-human AnxA2 monoclonal antibody diluted at 1:2000 was coated on a 96-well microtiter plate and was incubated overnight at 4°C. After blocking with 10% fetal bovine serum at room temperature for 1 h, an AnxA2 standard (recombinant human AnxA2 expressed by *Pichia pastoris*[24]) and test samples were added into each well and incubated at 37°C for 1 h. Rabbit anti-human AnxA2 polyclonal antibody was added and incubated at 37°C for 1 h. After the plate was washed three times, peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody was added

and incubated at 37°C for 1 h. AF-AnxA2 was detected with *o*-phenylenediamine. Hydrochloric acid (1M) was then added to stop the reaction. The absorbance of the solution was measured at 492 nm using a microplate spectrofluorometer. The concentration of AnxA2 in the samples was calculated from the AnxA2 standard curve. AF-IL-8 was measured with ELISA as previously reported.[26]

Statistical analysis

The data were analyzed using the SPSS 11.0 software for Windows. Simple linear regression analysis was conducted to evaluate the correlation between AF-AnxA2 and gestational age and between AF-AnxA2 and AF-IL-8. A cohort study was conducted to compare patients who developed hCAM with the remainder of the cohort. hCAM patients and the remainder were compared with respect to baseline and outcome characteristics using appropriate parametric and non-parametric statistical tests. Differences in proportions were assessed using the χ^2-test. AF-AnxA2 was compared between hCAM patients and the remainder of the cohort and between pPROM patients and the remainder using the Mann–Whitney *U*-test. Logistic regression analysis, adjusting for the potential confounding clinical factors associated with hCAM or pPROM, was conducted to evaluate the independent association of AF-AnxA2 with hCAM or pPROM. The strength of association in these models is reported as the adjusted odds ratio (OR) with the 95% confidence interval (CI). Receiver–operator characteristic (ROC) curve analysis was used to determine an optimal AF-AnxA2 cut-off point for predicting preterm delivery within 2 weeks of sampling. The χ^2-test with specific cut-off levels was analyzed, and the relative risk was calculated to estimate whether patients with an AF-AnxA2 greater than the cut-off level have a risk of having preterm delivery. Each group categorized by cut-off levels of AF-AnxA2 and AF-IL-8 was compared with respect to sampling-to-delivery interval using the Mann–Whitney *U*-test. A *P*-value of <0.05 indicated significance.

Results

Baseline and outcome characteristics of hCAM patients and the remainder of the cohort

Fifty-five patients agreed to participate. The mean and median gestational ages at the time of sampling were 26.5 (standard deviation: 3.2) and 26.4 (range: 20.3–31.9) weeks, respectively. Of the 55 patients, there were 38 patients with hCAM (69.1%). The other 17 patients (30.9%) served as the comparison group. Baseline and outcome characteristics were compared between hCAM patients and the remaining cohort. No significant differences in maternal age, gestational ages at the time of sampling and delivery, sampling methods, number of nulliparous patients, birth weights, sampling-to-delivery intervals, and incidence of pregnancy complications were observed between hCAM patients and the remainder of the cohort (Table 1).

AF-AnxA2

The correlation of AF-AnxA2 with gestational age is shown in Figure 1. AF-AnxA2 significantly decreased as the gestational age at the time of sampling increased between 20 and 32 weeks (simple linear regression: $\beta = -0.283$, $P = 0.036$; Fig. 1) in patients diagnosed with preterm labor and/or pPROM.

AF-AnxA2 was higher in hCAM patients than in the comparison group, but the difference was not statistically significant ($P = 0.053$; Fig. 2a). We also tested eight patients who did not have preterm labor or pPROM (mean gestational age ± standard deviation: 36.6 ± 1.6 weeks; range: 34.1–38.6 weeks) for the presence of AF-AnxA2, but it was not detected in these patients. In Figure 2a, open circles indicate patients who developed pPROM at the time of admission. We compared AF-AnxA2 between pPROM patients ($n = 16$) and non-pPROM patients ($n = 39$) in the present cohort, and AF-AnxA2 for pPROM patients was significantly higher than that for non-pPROM patients ($P = 0.002$; Fig. 2b).

Tables 2 and 3 display OR for the strength of association between hCAM or pPROM and their risk factors. Positivity for AF-AnxA2 (>878.2 ng/mL) and AF-IL-8 (>13.3 ng/mL) were significantly associated with pPROM (OR, 5.61; 95%CI, 1.38–22.88; and OR, 4.31; 95%CI, 1.18–15.81, respectively). The association between positivity for AF-AnxA2 and pPROM remained significant after adjustment for confounding factors in logistic regression analyses (OR, 6.52; 95%CI, 1.18–36.10).

Biomarker for prediction of preterm delivery

We sought to dichotomize AF-AnxA2 to evaluate the association of elevated levels with subsequent preterm delivery within 2 weeks of sampling. ROC curve analysis of the area under the curve (68%) indicated that AF-AnxA2 is a fair test for predicting preterm delivery

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Table 1 Baseline and outcome characteristics of the cohort†

Baseline characteristics	hCAM patients ($n = 38$)	Remaining cohort ($n = 17$)	*P*-value
Maternal age (years)	30.1 ± 4.4	30.0 ± 5.6	0.955‡
	30 (21–38)	28 (23–40)	0.674§
Gestational age at sampling (weeks)	26.3 ± 3.2	26.8 ± 3.2	0.628‡
	26.3 (20.3–31.9)	27.3 (21.1–31.6)	0.572§
Sampling			
Amniocentesis	92%	88%	0.963¶
Cesarean section	8%	12%	0.963¶
Parity			
Nulliparous	41%	59%	0.338¶
Pregnancy complications			
Pre-eclampsia	0%	6%	
pPROM	37%	12%	0.116¶
Birthweight (g)	1444 ± 717	1569 ± 631	0.547‡
	1218 (440–2888)	1412 (758–2670)	0.454§
Gestational age at delivery (weeks)	29.6 ± 4.4	30.4 ± 4.5	0.515‡
	28.9 (21.9–40.3)	31.0 (25.4–37.9)	0.444§
Sampling-to-delivery interval (days)	22.5 ± 28.9	25.3 ± 28.0	0.742‡
	9 (0–105)	19 (0–83)	0.647§

†Data presented as mean ± standard deviation, median (range), and/or percentage. ‡Parametric *t*-test. §Non-parametric Mann–Whitney *U*-test. ¶χ^2-test for categorical data. hCAM, histological chorioamnionitis; pPROM, preterm premature rupture of the membranes.

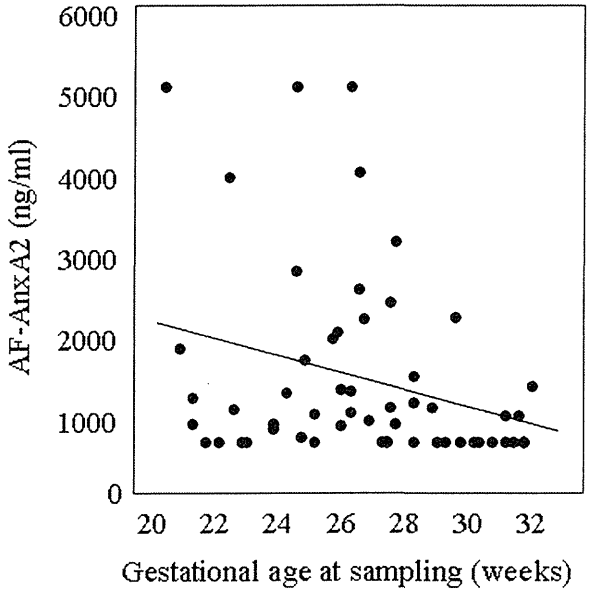

Figure 1 Scatter plot of amniotic fluid–annexin A2 (AF-AnxA2) versus gestational age at sampling with associated regression line. AF-AnxA2 significantly decreased as the gestational age at the time of sampling increased (simple linear regression, $\beta = -0.283$, $P = 0.036$).

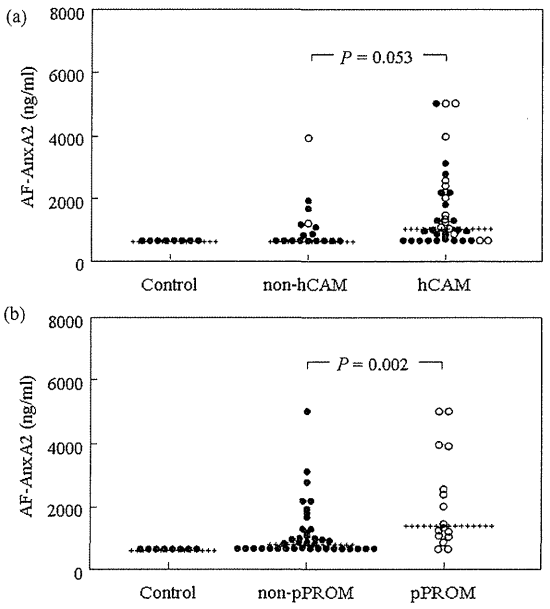

Figure 2 Dot plot of amniotic fluid–annexin A2 (AF-AnxA2) for (a) histological chorioamnionitis (hCAM) patients versus non-hCAM patients and (b) for preterm premature rupture of the membranes (pPROM) patients versus non-pPROM patients. (a) AF-AnxA2 was higher in hCAM patients than in non-hCAM patients ($P = 0.053$). (b) AF-AnxA2 was significantly higher in pPROM patients than in non-pPROM patients ($P = 0.002$). Open circles indicate patients who developed pPROM at the time of admission.

Table 2 Risk factors for hCAM

	Univariate		Multivariate	
	OR	95%CI	OR	95%CI
AF-AnxA2 (>878.2 ng/mL)	3.14	0.95–10.37	2.326	0.62–8.74
AF-IL-8 (>13.3 ng/mL)	2.52	0.77–8.25	1.748	0.44–6.97
Maternal age (>35 years)	0.88	0.19–4.01	0.528	0.09–3.08
Nulliparous	0.48	0.15–1.53	0.579	0.15–2.18
pPROM	4.38	0.87–22.02	2.270	0.38–13.67

AF, amniotic fluid; AnxA2, annexin A2; CI, confidence interval; hCAM, histological chorioamnionitis; IL-8, interleukin 8; OR, odds ratio; pPROM, preterm premature rupture of the membranes.

Table 3 Risk factors for pPROM

	Univariate		Multivariate	
	OR	95%CI	OR	95%CI
AF-AnxA2 (>878.2 ng/mL)	5.61	1.38–22.88	6.52	1.18–36.10
AF-IL-8 (>13.3 ng/mL)	4.31	1.18–15.81	5.11	0.99–26.34
Maternal age (>35 years)	1.27	0.28–5.85	0.75	0.12–4.82
Nulliparous	0.70	0.21–2.35	1.24	0.27–5.57
hCAM	4.38	0.87–22.02	2.59	0.42–15.90

AF, amniotic fluid; AnxA2, annexin A2; CI, confidence interval; hCAM, histological chorioamnionitis; IL-8, interleukin 8; OR, odds ratio; pPROM, preterm premature rupture of the membranes.

within 2 weeks (Fig. 3). An AF-AnxA2 of 878.2 ng/mL was chosen as the cut-off level, which resulted in a sensitivity of 68.8%, a specificity of 65.2%, a positive predictive value (PPV) of 73.3%, and a negative predictive value (NPV) of 60.0% (Table 4). An AF-AnxA2 > 878.2 ng/mL indicated a relative risk of 1.8 for the development of preterm delivery within 2 weeks of sampling (χ^2, $P = 0.026$). In other words, patients with an AF-AnxA2 > 878.2 have a risk of developing preterm delivery within 2 weeks of sampling that is 1.8 times the risk in those with a value <878.2 (data not shown).

Predictive value of the combination of AF-AnxA2 and AF-IL-8

We have previously reported that an AF-IL-8 > 13.3 ng/mL in pregnant women in preterm labor with intact membranes is a highly predictive marker for detecting preterm delivery before 34 weeks of gestation,[8] so we investigated the correlation between

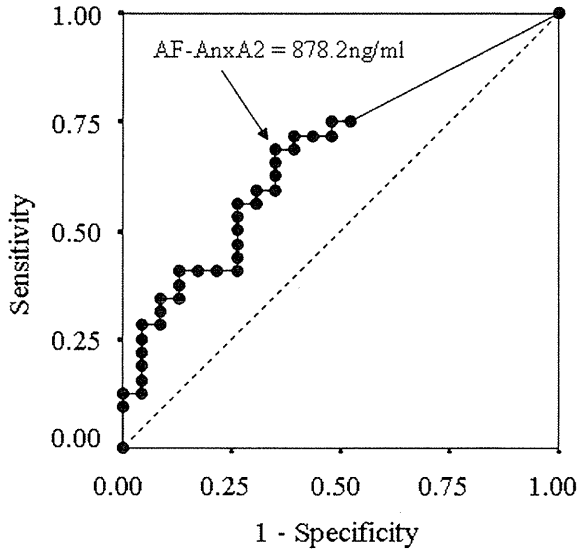

Figure 3 Receiver–operator characteristic curve indicating the ability of amniotic fluid–annexin A2 (AF-AnxA2) to predict preterm delivery within 2 weeks of sampling. The area under the curve was 68%. At a cut-off level of 878.2 ng/mL, the sensitivity was 68.8%, the specificity was 65.2%, the positive predictive value was 73.3%, and the negative predictive value was 60.0%.

AF-AnxA2 and AL-IL-8, and whether the combination of AF-AnxA2 and AF-IL-8 was a better predictive marker for preterm delivery in patients with preterm labor or pPROM than either alone. We used an AF-AnxA2 of 878.2 ng/mL and an AF-IL-8 of 13.3 ng/mL as cut-off levels for predicting preterm delivery within 2 weeks of sampling. AF-AnxA2 was significantly correlated with AF-IL-8 (simple linear regression: $\beta = 0.407$, $P = 0.002$; Fig. 4a). The combination of AF-AnxA2 and AF-IL-8 resulted in a sensitivity of 53.1%, a specificity of 91.3%, a PPV of 89.5%, and an NPV of 58.3% (Table 4). Furthermore, sampling-to-delivery intervals were significantly shorter (median: 1.0 day) in patients with both an AF-AnxA2 > 878.2 ng/mL and an AF-IL-8 > 13.3 ng/mL (AnxA2+/IL-8+) than those in the other groups: AnxA2−/IL-8− (28.5 days), AnxA2−/IL-8+ (13.0 days), and AnxA2+/IL-8− (35.0 days) ($P < 0.01$; Fig. 4b).

Discussion

Members of the annexin family of proteins are involved in the anti-inflammatory response.[10] Annexin A1 has

Table 4 Diagnostic indices of AF tests in prediction of preterm delivery within 2 weeks of sampling

Variable (cut-off value)	Sensitivity	Specificity	PPV	NPV
AF-AnxA2 (>878.2 ng/mL)	68.8% (22/32)	65.2% (15/23)	73.3% (22/30)	60.0% (15/25)
AF-IL-8 (>13.3 ng/mL)	68.8% (22/32)	73.9% (17/23)	78.6% (22/28)	63.0% (17/27)
AF-AnxA2 & -IL-8	53.1% (17/32)	91.3% (21/23)	89.5% (17/19)	58.3% (21/36)

AF, amniotic fluid; AnxA2, annexin A2; IL-8, interleukin 8; NPV, negative predictive value; PPV, positive predictive value.

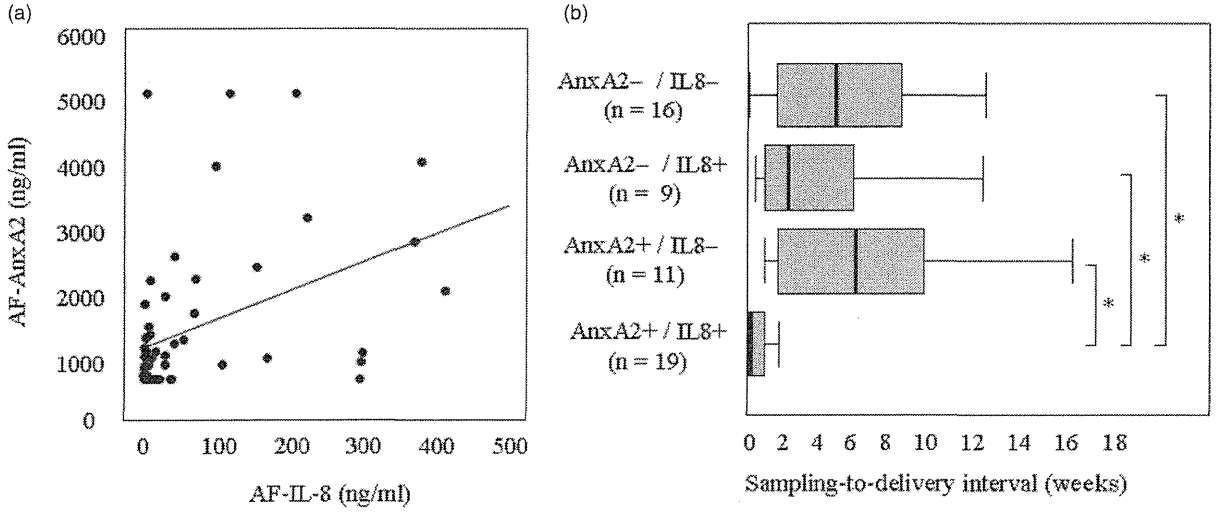

Figure 4 (a) Correlation between amniotic fluid–annexin A2 (AF-AnxA2) and AF level of interleukin-8 (AF-IL-8). (b) Sampling-to-delivery interval in groups categorized by cut-off levels of AF-AnxA2 and AF-IL-8. (a) AF-AnxA2 correlated significantly with AF-IL-8 (simple linear regression: $\beta = 0.407$, $P = 0.002$). (b) Sampling-to-delivery intervals in patients with AF-AnxA2 > 878.2 ng/mL and AF-IL-8 > 13.3 ng/mL (AnxA2+/IL-8+) were significantly shorter (median: 1.0 day) than in the remaining cohort: AnxA2–/IL-8– (28.5 days), AnxA2–/IL-8+ (13.0 days), and AnxA2+/IL-8– (35.0 days) ($P < 0.01$).

been clearly shown to mitigate anti-inflammatory events downstream of glucocorticoid induction, as well as to inhibit arachidonic acid production via direct inhibition of phospholipase A2.[27] The AnxA2 tetramer serves as a docking protein or recognition element for bacterial and viral pathogens, such as *Pseudomonas aeruginosa*,[11] cytomegalovirus,[12] respiratory syncytial virus,[13] and macrophage-tropic HIV.[14] Furthermore, soluble AnxA2 tetramer activates human macrophages via mitogen-activated protein kinases that produce tumor necrosis factor alpha, interleukin (IL)-1 beta, and IL-6.[28] These data indicate the participation of AnxA2 in microbial clearance and establishment of inflammation. The serum level of soluble AnxA2 tetramer is low in healthy people, but is elevated in patients with an infection.[29] We previously reported that AnxA2 is a target for fetal IgM autoantibody in preterm infants, and that the anti-AnxA2 IgM titer is associated with severity of placental inflammation, that is, hCAM.[22] Therefore, in the present study, we examined whether AF-AnxA2 is a novel marker for detecting intrauterine inflammation, which is the leading cause of preterm delivery.

AF-AnxA2 in patients with preterm labor or pPROM was measurable with the sandwich ELISA developed by us. AF-AnxA2 decreased significantly as the gestational age progressed from 20 to 32 weeks. This correlation between AF-AnxA2 and gestational age seems to be implicated in the incidence of hCAM in the causative factors leading to pPROM and preterm delivery. Significantly, the earlier the gestational age at which patients present with preterm labor, the higher the frequency of intrauterine infection. At 21–24 weeks of gestation, most spontaneous births are associated with hCAM compared with about 10% at 35–36 weeks of gestation.[30]

AF-AnxA2 was compared between hCAM patients and the remaining cohort to examine the association of AF-AnxA2 with hCAM. AF-AnxA2 in hCAM patients tended to be higher than that in non-hCAM patients ($P = 0.053$). We next examined the association between AF-AnxA2 and the rupture of the chorioamniotic membrane, pPROM. AF-AnxA2 was significantly higher in patients with pPROM than in the remaining cohort ($P = 0.002$). AnxA2 protein is expressed strongly in the chorioamniotic membrane, especially in amniotic epithelial cells during gestation.[23,24] Therefore, the significant difference in AF-AnxA2 between the pPROM patients and the remaining cohort might be implicated in the expression and localization of AnxA2 in the human placenta.

ROC curve analysis was performed to evaluate the significance of AF-AnxA2 as a novel marker for predicting subsequent preterm delivery. The results showed that an AF-AnxA2 > 878.2 ng/mL was an acceptable marker for subsequent preterm delivery within 2 weeks of sampling (area under the curve: 68%). However, it was not a substantially superior, sole predictive marker for preterm delivery compared to the previously reported markers, such as AF IL-6, IL-8, glucose, and white blood cell count.[8] Thus, we next examined the importance of the combination of AF-AnxA2 and AF-IL-8 as a predictor of preterm delivery. This combination, compared with AF-AnxA2 alone, dramatically improved the specificity from 65.2 to 91.3% and the PPV from 73.3 to 89.5%. The gestational duration was shorter in patients with high AF-AnxA2 and AF-IL-8 than in those with lower levels. Moreover, 84% (16/19) of patients with high AF-AnxA2 and AF-IL-8 delivered within 1 week of sampling.

Our data indicate that AF-AnxA2 is a potential temporal biomarker for predicting preterm delivery. The secretion or release mechanism of AnxA2 from membranes into amniotic fluid is not completely clear. An increase in collagenolysis and a decrease in membrane collagen content by activation of MMP have been documented in patients with pPROM.[31] MMP activation results in increased fetal membrane apoptosis, which subsequently results in further increase in MMP activation.[32] The above findings suggest the involvement of AnxA2 secretion/release with the activation of MMP: (i) the membrane-anchored AnxA2 might be directly cleaved by MMP; and (ii) AnxA2 might be secreted concomitantly with the programmed cell death process of amniotic epithelial cells or chorionic trophoblasts.

In conclusion, we identified AF-AnxA2 as a marker for subsequent preterm delivery and pPROM, rather than for hCAM. The combination of AF-AnxA2 and AF-IL-8 may predict preterm delivery in patients with preterm labor or pPROM. Further research is clearly needed to determine the role of AnxA2 in placental inflammatory complications, and we are currently collecting data to accomplish this goal.

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Article

Apoptosis Signaling Is Altered in $CD4^+CD25^+FoxP3^+$ T Regulatory Lymphocytes in Pre-Eclampsia

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Abstract: The aim of our study was to estimate the surface expressions of CD95 (APO-1/Fas) antigen and the intracellular expressions of anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax in $CD4^+CD25^+FoxP3^+$ T regulatory lymphocytes (Tregs) as well as the percentage of $CD8^+CD28^+$ T cytotoxic cells in peripheral blood of patients with pre-eclampsia in comparison with healthy pregnant women in the third trimester of physiological pregnancy. Twenty-four women with pre-eclampsia and 20 normal third trimester pregnant women were included in the study. The lymphocytes were isolated from peripheral blood samples and labeled with monoclonal antibodies. The expressions of surface antigens and intracellular proteins were estimated using flow cytometry. The population of $CD4^+CD25^+FoxP3^+$ Treg cells was significantly lower in peripheral blood of patients with pre-eclampsia when compared to normal third trimester pregnant women. The percentages of $CD4^+CD25^+FoxP3^+$ Treg cells that express Bcl-2 protein were significantly lower in peripheral blood of patients with pre-eclampsia when compared to healthy pregnant women, whereas the percentages of $CD4^+CD25^+FoxP3^+$ Treg cells with the expressions of Bax protein did not differ in both groups. Moreover, the mean

fluorescence intensity (MFI) of Bcl-2 protein in $CD4^+CD25^+FoxP3^+$ Treg cells was significantly lower and MFI of Bax protein significantly higher in pre-eclampsia when compared to the control group. The percentage of $CD8^+CD28^+$ T cells did not differ in both studied groups but MFI of CD28 antigen on T $CD8^+$ cells was significantly higher in pre-eclampsia when compared to the control group. The obtained results suggest that the deficit of $CD4^+CD25^+FoxP3^+$ Treg lymphocytes which is observed in pre-eclampsia may be associated with altered apoptosis signaling in Tregs.

Keywords: apoptosis; Bax protein; Bcl-2 protein; CD95 antigen; pre-eclampsia; pregnancy; Treg cells

1. Introduction

Pre-eclampsia (PE) is a common obstetric syndrome affecting about 5–10% of pregnant women [1]. The etiology and pathogenesis of this syndrome are not fully understood. There are many studies describing alterations in the innate and adaptive immune system which may have an influence on the onset of this disorder. It was suggested that the activation of cell-mediated immunity may play the key role in the etiology of pre-eclampsia. It was proposed that inappropriate activation of the immune system can lead to pre-eclampsia [1–4].

Regulatory T lymphocytes $CD4^+CD25^{bright}$ (Tregs) are known to play an important role in the development and maintenance of tolerance in peripheral tissues [5,6]. It has been demonstrated that Treg cells have a role in induction of transplantation tolerance. They express high level of CD25 (IL-2Rα) as well as cytotoxic T lymphocyte antigen 4 (CTLA-4) and the transcription factor Foxp3 [5–10].

Maternal immune system responses to fetal antigens play a critical role [11]. There is some evidence that T regulatory lymphocytes (Treg cells) play an essential role in controlling and preventing fetal rejection. It was suggested that high levels of Treg cells are extremely important for successful pregnancy [12–15]. Furthermore, was observed that the populations of peripheral blood and decidual Treg cells are reduced in pre-eclampsia [16–19]. On the other hand, there were reports that the numbers of peripheral blood Tregs were similar in pre-eclampsia and normal pregnancy [20,21]. However, it should be taken into account that in those studies the sample numbers were small or the authors estimated $CD4^+CD25^+$ T cells, and they did not evaluate $CD4^+CD25^{high}$ Treg cells [20,21].

Treg cells cooperate with $CD8^+CD28^-$ T lymphocytes which have also a regulatory capacity [22]. On the other hand, the expressions of CD28 antigens on $CD8^+$ T cells enhance a T cell activation which may lead to immune responses at the fetal-maternal interface [23].

Apoptosis (programmed cell death) is an important immunoregulatory mechanism for maintaining homeostasis in the immune system. There are a few pathways controlling programmed cell death. The protein Bcl-2 is one of the factors which regulates apoptosis [24,25]. In humans the family of apoptotic proteins consists of more than 15 members divided into two groups: anti-apoptotic and pro-apoptotic proteins. The proteins: Bcl-2 and Bcl-xLong are characterized as anti-apoptotic proteins thus preventing cell death. The proteins: Bax, Bak, Bad, Bcl-xShort and Bid are known as pro-apoptotic proteins [24,25].

Activated lymphocytes are removed by Fas/FasL mechanism called activation induced cell death (AICD) [26,27]. The molecule CD95 (APO-1/Fas) is a surface antigen detectable on activated lymphocytes. Thus, activated lymphocytes may be able to undergo Fas/Fas-L-mediated apoptosis process independently of the Bcl-2 protein family. The antigen CD95 (APO-1/Fas) belongs to the tumor necrosis factor-receptor family (TNF-R).

It was reported recently that the proportion of peripheral blood Treg cells decreases in preeclampsia, but it is unclear why Treg cells decrease [16–19].

The aim of our study was to evaluate the levels of $CD4^+CD25^+FoxP3^+$ Treg cells and $CD8^+CD28^+$ T lymphocytes in peripheral blood of patients with pre-eclampsia and healthy pregnant women in the third trimester of normal pregnancy. Furthermore, the aim of the study was to evaluate the surface expressions of CD95 antigen and intracellular expressions of Bcl-2 and Bax proteins in $CD4^+CD25^+FoxP3^+$ Treg cells of patients with pre-eclampsia and normal third trimester pregnant women.

2. Results and Discussion

The population of $CD4^+CD25^+FoxP3^+$ Treg cells was significantly lower in peripheral blood of patients with pre-eclampsia when compared to healthy normotensive pregnant women in the third trimester of normal pregnancy ($p < 0.05$). The results are presented in the Table 1.

Table 1. The percentage of $CD4^+CD25^+FoxP3^+$ Treg cells as well as the expressions of CD95, Bax and Bcl-2 antigens on $CD4^+CD25^+FoxP3^+$ Treg cells in peripheral blood of patients with pre-eclampsia and health third trimester pregnant women.

	Patients with pre-eclampsia ($n = 24$) median and interquartile ranges	**Normal third trimester pregnancy ($n = 20$) median and interquartile ranges**	*p*
$CD4^+CD25^+FoxP3^+$ Treg cells	3.60% (2.30%–6.01%)	6.20% (5.15%–7.60%)	$p < 0.05$
CD95 antigen on $CD4^+CD25^+FoxP3^+$ Treg cells	63.34% (49.38%–68.60%)	64.32% (51.90%–72.89%)	NS
Bcl-2 protein on $CD4^+CD25^+FoxP3^+$ Treg cells	69.10% (52.00%–88.00%)	97.45% (95.70%–99.30%)	$p < 0.05$
Bax protein on $CD4^+CD25^+FoxP3^+$ Treg cells	21.15% (19.59%–28.95%)	23.97% (19.77%–30.66%)	NS
$CD8^+CD28^+$ T cells	6.50% (3.59%–12.85%)	6.75% (4.07%–9.41%)	NS

There were no significant differences in the percentages of $CD4^+CD25^+FoxP3^+$ Treg cells with the expressions of APO-1/FAS (CD95) antigen in peripheral blood of patients with pre-eclampsia and normal third trimester pregnant women. Similarly, the mean fluorescence intensity (MFI) of APO-1/FAS (CD95) antigen on $CD4^+CD25^+FoxP3^+$ Treg cells did not differ in peripheral blood of patients with pre-eclampsia when compared to the control group. The results are presented in Tables 1 and 2.

Table 2. The mean fluorescence intensity (MFI) of CD95, Bax and Bcl-2 antigen on $CD4^+CD25^+FoxP3^+$ Treg cells as well as MFI of CD28 antigen on T $CD8^+$ cells in peripheral blood of patients with pre-eclampsia and healthy third trimester pregnant women.

	Patients with pre-eclampsia ($n = 24$) median and interquartile ranges (arbitral units)	**Healthy third trimester pregnant women ($n = 20$) median and interquartile ranges (arbitral units)**	*p*
MFI CD95 antigen on $CD4^+CD25^+FoxP3^+$ cells	52.05 a.u. (46.93 a.u.–56.62 a.u.)	49.41 a.u. (42.79 a.u.–55.60 a.u.)	NS
MFI Bax antigen on $CD4^+CD25^+FoxP3^+$ cells	185.34 a.u. (150.51 a.u.–206.13 a.u.)	102.76 a.u. (86.13 a.u.–124.15 a.u.)	$p < 0.01$
MFI Bcl-2 antigen on $CD4^+CD25^+FoxP3^+$ cells	2343 a.u. (2077.00 a.u.–2592 a.u.)	2800.00 a.u. (2315.00 a.u.–3858.00 a.u.)	$p < 0.05$
MFI CD28 antigen on T $CD8^+$ cells	131.26 a.u. (98.47 a.u.–146.14 a.u.)	63.34 a.u. (68.56 a.u.–88.95 a.u.)	$p < 0.001$

The percentages of peripheral blood $CD4^+CD25^+FoxP3^+$ Treg cells that express Bax protein were similar in both studied groups. However, MFI of Bax protein in $CD4^+CD25^+FoxP3^+$ Treg cells was significantly higher in peripheral blood of patients with pre-eclampsia when compared to the control group ($p < 0.01$). The results are presented in Tables 1 and 2.

The percentages of peripheral blood $CD4^+CD25^+FoxP3^+$ Treg cells that express Bcl-2 protein were significantly lower in patients with pre-eclampsia when compared to the control group ($p < 0.05$). Moreover, MFI of Bcl-2 protein in peripheral blood Treg cells of patients with pre-eclampsia was significantly lower when compared to normal third trimester pregnant women ($p < 0.05$). The results are presented in Tables 1 and 2.

The percentages of peripheral blood $CD8^+CD28^+$ T cells did not differ in the group of patients with pre-eclampsia when compared to the healthy pregnant women. However, the MFI of CD28 antigen on $CD8^+$ T cells was significantly higher in the group of patients with pre-eclampsia when compared to the control group ($p < 0.001$). The results are presented in Tables 1 and 2.

During physiological pregnancy there are a lot of alterations in the maternal immune system which prevent an inappropriate response against fetal antigens. Many studies confirmed the theory concerning "Th2 phenomenon", which claimed that during normal pregnancy there is a predominance of Th2 over Th1 type immunity [28]. In the following years "Th2 phenomenon" was revised [29]. In recent studies, it was hypothesized that Treg cells play a crucial role in the maternal tolerance to fetal antigens. This is possible because of their capacity to regulate the activation of allo-reactive T cells [12–15]. It was observed that normal human pregnancy is associated with the expansion of Treg cells [12–15]. On the other hand, in the cases of patients with pre-eclampsia decreased levels of Treg cells were found [16–19].

Although the factors regulating $CD4^+CD25^+$ Treg cells have not been fully defined yet, there are some reports suggesting that estrogens may modify maternal immune response. Polanczyk *et al.* demonstrated on animal models that the treatment with estrogens up-regulates $CD4^+CD25^+$ T cell subset [30]. It was observed that the concentrations of estrogens are lower in pre-eclampsia when

compared to physiological pregnancy [31]. Therefore, it seems possible that the population of Treg cells is decreased in that mechanism in pre-eclampsia.

On the other hand, it has been observed that in pre-eclampsia the production of Interleukin-1β (IL-1β) and Interleukin-6 (IL-6) is increased [32]. It is known that the secretion of IL-6, which is a pro-inflammatory cytokine, suppresses the protective immunoregulatory properties of $CD4^+CD25^+$ Treg cells [33]. It seems possible that the increased levels of IL-6 in pre-eclampsia can suppress the expansion of Treg cells in this syndrome.

It has been shown in recent studies that Th17 cells, which induce inflammatory response and Treg cells, which have regulatory properties are developmentally linked [34]. Interleukin-1β and IL-6 induce the differentiation of naïve T cells into Th17 cells, and these cytokines could induce the development of Th17 cells from Treg cells [34,35]. Transforming growth factor-β (TGF-β) is known to drive the differentiation of Treg cells and to inhibit the differentiation of Th17 cells [36]. In pre-eclampsia the differentiation of Treg cells might be decreased resulting in small numbers of Tregs because of elevated levels of soluble endoglin, which is known to inhibit the TGF-β signalling [37]. In fact, Santner-Nanan *et al.* reported in their study that the population of Th17 cells is increased and the population of Treg cells is decreased in pre-eclampsia [18].

Finally, the deficit of Treg cells in pre-eclampsia might be explained by the susceptibility to apoptosis in Treg cells. Fas/FasL-mediated apoptosis seems to be the most important mechanism to keep appropriate balance during pregnancy. Normally, anti-apoptotic protein Bcl-2 remains unchanged during normal pregnancy [37].

It has been noticed that the induction of Fas/Fas-L mechanism can lead to the depletion of Treg lymphocytes [30]. It was found that APO-1/Fas (CD95) antigen is highly expressed on $CD4^+CD25^+FoxP3^+$ Treg cells. Treg cells are highly susceptible to the ligand for CD95 (CD95L), but not to T cell receptor mediated cell death [38].

The family of Bcl-2 protein is composed of both pro- and anti-apoptotic proteins. It has been suggested that progesterone modulates the expressions of Bcl-2 and Bax proteins. Low concentrations of progesterone lead to the down-regulation of Bax protein and increased levels of Bcl-2 protein [38]. The protein Bcl-2 is described as an inhibitor of programmed cell death, it down-regulates cytochrome c mitochondrial release notably by impairing the pro-apoptotic function of Bax protein. In contrast, the protein Bax releases cytochrome c from mitochondria which induces apoptosis through the activation of caspases 3, 6, 7, 9 [24,25].

In our study we observed the deficit of $CD4^+CD25^+FoxP3^+$ Treg cells in peripheral blood of patients with pre-eclampsia when compared to healthy normotensive pregnant women in the third trimester of normal pregnancy. We hypothesized that the alterations in apoptotic mechanisms are responsible for decreased levels of Treg lymphocytes in pre-eclampsia. We revealed significantly lower percentages of peripheral blood $CD4^+CD25^+FoxP3^+$ Treg cells that express Bcl-2 protein as well as significantly lower MFI of Bcl-2 protein and higher MFI of Bax protein in Treg cells of pre-eclamptic patients in comparison with normal third trimester pregnant patients. The results may suggest that apoptosis of activated $CD4^+CD25^+FoxP3^+$ Treg cells is up-regulated in pre-eclampsia, although we did not show the direct evidence of apoptosis of Treg cells of pre-eclamptic patients. It is very difficult to show the apoptotic peripheral blood cells, because these cells are eliminated by

phagocytosis. Indeed, there are no papers that show apoptotic peripheral blood cells in cases of obstetric complications of human pregnancy.

We did not find any differences in the expressions of CD95 (APO-1/Fas) antigen on peripheral blood $CD4^+CD25^+FoxP3^+$ Treg cells of patients with pre-eclampsia and normal pregnant patients. The results suggest that the deficit of Treg cells in pre-eclampsia is not associated with the alterations in the expressions of CD95 antigen.

In the present study we also evaluated the expressions of CD28 antigen on peripheral blood $CD8^+$ T lymphocytes of patients with pre-eclampsia and normal third trimester pregnant women. The antigen CD28 is thought to be a co-stimulatory molecule which may enhance T-cells activation [22,39]. We studied the percentages of T $CD8^+CD28^+$ lymphocytes but we did not find any differences between the populations of these cells in normal pregnancy and pre-eclampsia. However, the mean fluorescence intensity of CD28 antigen on T $CD8^+$ lymphocytes was significantly higher in pre-eclampsia when compared to normal third trimester pregnant women. The results suggest the role of CD28 antigen in T cell activation in the pathogenesis of pre-eclampsia.

The crucial influence of CD28 and CTLA-4 (cytotoxic T-lymphocyte antigen-4) antigens on T-cell immune response has been known for over a decade. Initially viewed as molecules that provide intracellular stimulatory and inhibitory signals, recent evidence suggests that both of them are also important in the homeostasis and function of a regulatory T cells (Treg). The antigens CD28 and CTLA-4 play an important role in the development of T cells in thymus and both can influence thymic selection of T cells. They have an impact on positive/negative selection of Treg and CD28 signaling regulates expression of Foxp3 in the thymocytes. These two molecules are also important in generation and regulation of Tregs in peripheral tissues. Tregs may utilize CTLA-4 (or possibly CD28) expression to increase expression of IDO (indoleamine 2,3-dioxygenase) in dendritic cells (DC). This may exert a dominant suppressive effect by depleting the environment of tryptophan. The antigen CD28 may provide signals for Treg proliferation as well as survival. The antigen CTLA-4 is implicated in the suppressive function of Tregs. The disruption of the interaction of CTLA-4 with its ligands can downregulate the immunosupressive activity of Treg. Treg interactions with DC may alter the adhesion or motility of CD25 negativeT lymphocytes and it relate to a role proposed for CTLA-4 in T-cell adhesion [39].

3. Material and Methods

3.1. Patients

The patients participating in this study were admitted to the Department of Obstetrics and Perinatology, Medical University of Lublin, Poland. The study group consisted of 24 pregnant women with pre-eclampsia. They were admitted to the hospital because of the symptoms of the disease but not because of signs of labour. Pre-eclamptic patients were classified according to the regulations of the Committee on Terminology of *American College of Obstetricians and Gynecologists*. Pre-eclampsia was characterized by blood pressure of at least 140/90 mmHg and proteinuria above 0.3 g/24 h. None of the pre-eclamptic patients was affected by preexisting clinical disorders, such as: chronic hypertension, renal diseases before pregnancy, and none of the pregnancies was complicated by

preterm labor or chorioamnionitis. The control group included 20 healthy normotensive pregnant women in the third trimester of normal pregnancy. They were recruited from the outpatient clinic. All pregnancies from the study and control groups were single. The patients from the study and control groups were not in active labor. The characteristics of the study and control groups are presented in Table 3. The study design was accepted by the local Ethics Committee. Informed consent for peripheral blood sampling was obtained from the patients.

Table 3. The clinical characteristics of patients with pre-eclampsia (study group) and healthy normotensive women in the third trimester of uncomplicated pregnancy (control group).

	Study group mean ± SD $n = 24$	**Control group mean ± SD $n = 20$**	**Significance (p)**
Maternal age	28.68 ± 4.76	27.68 ± 5.31	NS
Gravidity	1.84 ± 1.12	1.93 ± 0.85	NS
Parity	1.63 ± 0.95	1.81 ± 0.81	NS
Time of blood collection (weeks of gestation)	34.05 ± 2.14	34.62 ± 1.63	NS
Systolic pressure (mmHg)	155.35 ± 15.85	110.25 ± 20.15	<0.01
Diastolic pressure (mmHg)	96.47 ± 5.66	73.56 ± 7.18	<0.05
Proteinuria (g/24 h)	1.45 ± 0.65	absent	-
Uric acid (mg/dL)	5.47 ± 1.38	3.15 ± 1.43	<0.01
Fetal weight (g)	2560 ± 615	3280 ± 365	<0.05

3.2. Blood Sampling and Cell Preparation

Twenty milliliters of blood from both pre-eclamptic patients and normal third trimester pregnant women were taken by venipuncture and collected in sterile heparinized tubes. Blood samples from the patients with pre-eclampsia were taken before any treatment, such as: the administration of antenatal steroids or antihypertensive drugs.

3.3. Isolation of Peripheral Blood Cells

Peripheral blood mononuclear cells (PBMCs) were separated by gradient centrifugation on the lymphocyte separation medium, Lymphoprep (Nycomed, Torshov, Norway). They were centrifuged for 30 min at 600× g at 4 °C, collected from the interface with a Pasteur pipette and washed twice by centrifugation for 5 min at 250 g at 4 °C in 2 mL of the buffer containing phosphate-buffered saline (PBS; Serum and Vaccine Factory, Biomed, Lublin, Poland) with 2% FCS (Fetal Calf Serum, GibcoBRL, Paisley, UK) and the total numbers of cells were determined using the microscope.

3.4. Phenotyping of T Cells

The cell surface and intracellular antigens were determined on the fresh cells at the time of the sample submission. The cells were stained according to the manufacturer's protocols. The cells were labeled by direct staining of monoclonal antibodies. Five hundred microliters of cell suspensions were added to 5 μL of appropriate solution of fluorescein isothiocyanate (FITC)-conjugated antibodies

(Dako, Denmark and Becton Dickinson, San Diego, CA, USA), phycoerythrin (PE)-conjugated antibodies (BioLegend, San Diego, CA, USA), PE-Cy5-conjugated antibodies (BioLegend, San Diego, CA, USA) and Pacific Blue-labeled antibodies (BioLegend, San Diego, CA, USA) in the following combinations:

1. anti-Human CD8 (FITC) and anti-Human CD28 (PE) monoclonal antibodies;
2. anti-Human CD95 (FITC), anti-Human CD25 (PE), anti-Human CD4 (PE-Cy5) and anti-Human FoxP3 (Pacific Blue) monoclonal antibodies;
3. anti-Human Bax (FITC), anti-Human CD25 (PE), anti-Human CD4 (PE-Cy5) and anti-Human FoxP3 (Pacific Blue) monoclonal antibodies;
4. anti-Human Bcl-2 Oncoprotein (FITC), anti-Human CD25 (PE), anti-Human CD4 (PE-Cy5) and anti-Human FoxP3 (Pacific Blue) monoclonal antibodies.

The mixture of cells and antibodies was incubated for 30 min at 4 °C in the dark, centrifuged, washed twice by adding 1 mL of cold PBS to each tube with 1% sodium azide and 1% FCS and centrifuged again at 400× g for 10 min. After the standard incubation with antibodies directed against surface markers, the incubation by fixation and permeabilisation with FoxP3 Fix/Perm Buffer and FoxP3 Perm Buffer (BioLegend, San Diego, CA, USA) was performed. The next step of the protocol was incubation with antibodies directed against intracellular proteins: FoxP3 and Bcl-2 or Bax. Next, the supernatant was separated and after washing each sample was suspended in 200 μL PBS. As the last step, cells were immediately analyzed with the use of Becton Dickinson Canto II flow cytometer (Becton Dickinson, San Diego, CA, USA) and analyzed with FACSDiva™ Software (Becton Dickinson, San Diego, CA, USA). The results were presented as percentage of cells stained with antibody. The percentage of positive cells was calculated by comparing with the control. Background fluorescence was determined using isotype-matched directly conjugated mouse anti IgG1/IgG2α monoclonal antibodies. The samples were gated on forward scatter versus side scatter to exclude debris and cell aggregates. To assess the number of places binding a monoclonal antibody and indirectly the number of antigens the Mean Fluorescence Intensity (MFI) was used. For the assessment of MFI, logarithmic units were applied with the use of FACSDiva™ Software (Becton Dickinson, San Diego, CA, USA). The Mean Fluorescence Intensity was measured in comparison to the upper limit of MFI of negative control. The representatives FACS plots of the expressions of Bcl-2 and Bax protein in T $CD4^+CD25^+FoxP3^+$ Treg cells in peripheral blood of one patient with pre-eclampsia are presented in Figures 1 and 2.

3.5. Statistical Analysis

Statistical differences between groups were estimated using a standard non-parametric test (Mann-Whitney U-test). The results were presented as a median and interquartile ranges. Differences were defined as statistically significant at the level of $p < 0.05$. Statistica 7.0 software (StatSoft Poland, Krakow, Poland) was applied to statistical analysis.

Figure 1. Flow cytometry analysis of the Bcl-2 antigen expression in $CD4^+CD25^+FoxP3^+$ cells. FITC—anti-Bcl-2, PE—anti-CD25, PE-Cy5—anti-CD4, Pacific Blue—anti-FoxP3. (**a**) gating of lymphocytes; (**b**) gating of $CD4^+$ cells; (**c**) gating of $CD4^+CD25^+$ cells; (**d**) analysis of $Bcl\text{-}2^+CD4^+CD25^+FoxP3^+$ (Q2-2) and $Bcl\text{-}2^+CD4^+CD25^+FoxP3^-$ (Q1-2).

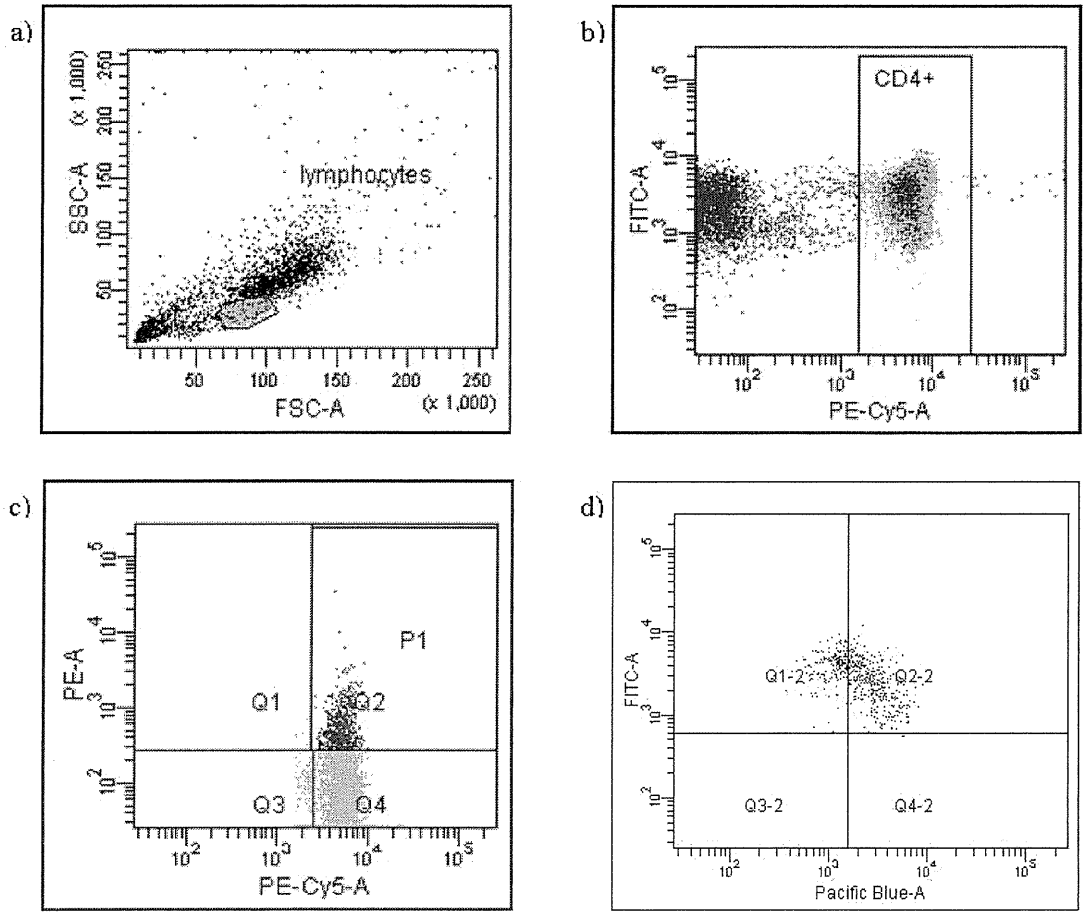


Figure 2. Flow cytometry analysis of the Bax antigen expression in $CD4^+CD25^+FoxP3^+$ cells. FITC—anti-Bax, PE—anti-CD25, PE-Cy5—anti-CD4, Pacific Blue—anti-FoxP3. (**a**) gating of lymphocytes; (**b**) gating of $CD4^+$ cells; (**c**) gating of $CD4^+CD25^+$ cells; (**d**) analysis of $Bax^+CD4^+CD25^+FoxP3^+$ (Q2-2) and $Bax^+CD4^+CD25^+FoxP3^-$ (Q1-2).

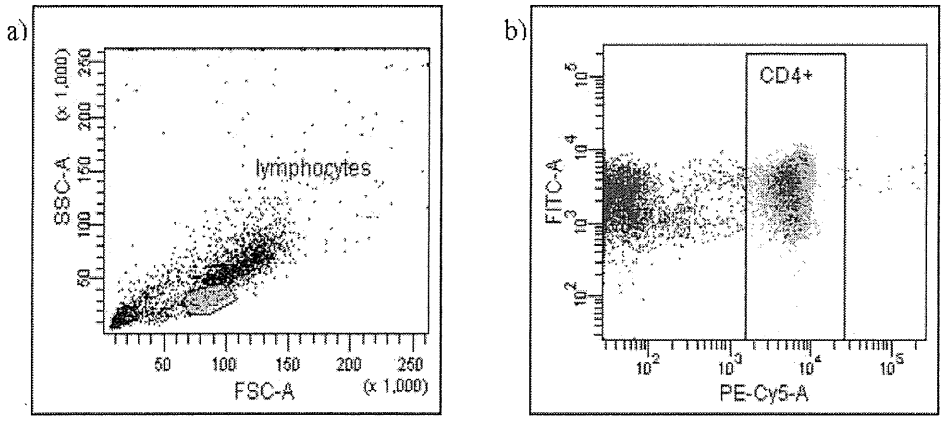
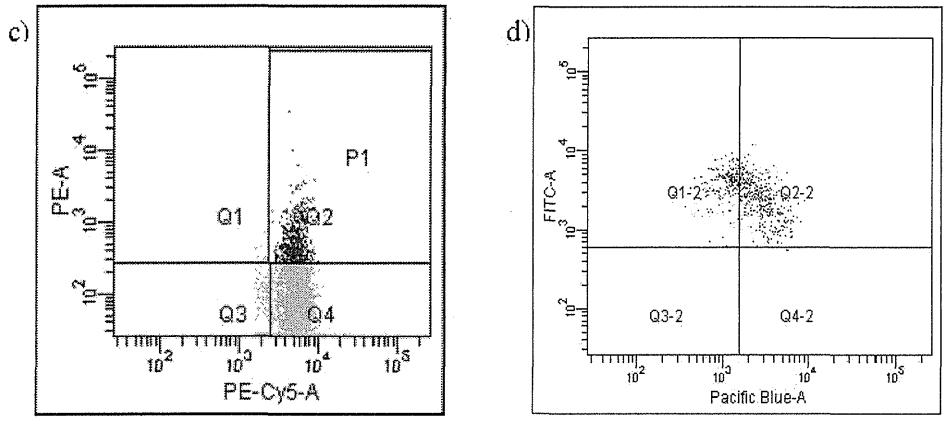


Figure 2. *Cont.*



4. Conclusions

The obtained results suggest that the deficit of $CD4^+CD25^+FoxP3^+$ Treg lymphocytes which is observed in pre-eclampsia may be associated with altered apoptosis signaling in Tregs.

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