Regular Article

Reduced ANXA5 mRNA and protein expression in pregnancies complicated by preeclampsia

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Abstract

Introduction: The placental anticoagulant protein Annexin A5 (ANXA5) is a multifunctional protein that is highly expressed on the apical surfaces of syncytiotrophoblasts, and plays an important role in haemostatic regulations, maintaining blood flow to the placenta. The aim of this study was to investigate the expression of ANXA5 in pregnancies complicated by preeclampsia (PE).

Materials and Methods: Placental tissue samples were collected from 23 pregnancies with PE and 34 normal pregnancies. ANXA5 mRNA levels were measured by quantitative Real-Time PCR (qPCR), while ANXA5 protein expression was measured by Western Blot (WB) and immunohistochemistry.

Results: ANXA5 mRNA expression in PE samples was lower than 1% of its expression in normal samples (mean ± SD: 0.002 ± 0.004 vs. 0.55 ± 0.38, p < 0.001), while ANXA5 protein levels in PE samples were approximately at 65% of the average normal expression (mean ± SD: 0.53 ± 0.30 vs. 0.81 ± 0.25, p = 0.001). Immunohistochemical analysis also verified the above results, since PE placentas tended to have low labelling indexes (LIs), in contrast to controls which demonstrated high LIs (p = 0.020). Statistical analysis of the WB data revealed that ANXA5 protein expression was increased in PE smokers vs. PE non-smokers (mean ± SD: 0.64 ± 0.23 vs. 0.41 ± 0.33, p = 0.027).

Conclusions: These results suggest that ANXA5 downregulation could be part of the pathophysiology of PE and the possible impairment in coagulation processes, which are seen in pregnancies that demonstrate PE. Further studies may investigate whether ANXA5 could be used as a biomarker for the early detection of PE and for the prediction of its severity.

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Introduction

Preeclampsia (PE) is a pregnancy-specific disorder characterized by hypertension and significant proteinuria developed at or after 20 weeks of pregnancy [1]. PE is a multisystem disease of widespread vascular endothelial malfunction and vasospasm, which complicates 2% of all pregnancies. It is a major cause of maternal and perinatal morbidity and mortality [1,2]. In particular, it is associated with adverse fetal outcomes, including fetal growth restriction (FGR), placental abruption, oligohydramnios and non-reassuring fetal surveillance. Although the exact pathophysiologic mechanism is not clearly understood, PE is primarily a disorder of placental dysfunction, leading to systemic endothelial dysfunction with associated vasospasm. In most cases, histopathologic examination of the placenta demonstrates evidence of placental insufficiency with associated abnormalities, such as placental infarction [3], retroplacental hematoma [4], blood infiltrates in the villous stroma, immature villi, increased fibrin deposition in perivillous space [5], and inflammatory placental decidual vasculopathy [6–11]. Abnormal trophoblastic invasion of the endometrium and impaired adaptation of the maternal decidua arterioles, as well as diffuse arterial microthrombosis, have been shown to underlie the process of abnormal placental and are considered essential in the development of PE [12].

The placentanticoagulant protein annexin A5 (ANXA5) is a member of the annexins, a family of calcium-dependent phospholipid-binding proteins. ANXA5 is a multifunctional protein; one of its proposed roles is the prevention of thrombosis. ANXA5, which is highly expressed on...
the apical surfaces of syncytiotrophoblasts, plays an important role in maintaining placental integrity in mice [13]. ANXA5 binds with high affinity in the presence of Ca\(^{2+}\) to negatively charged phospholipids, such as phosphatidylserine (PS), which is expressed on the external leaflet of the trophoblast membrane [14]. Cell membranes with PS exposed at the outside provide a catalytic surface for coagulation reactions, resulting in a high rate of prothrombinase complex formation and the activation of coagulation [15,16]. Therefore, ANXA5 forms an antithrombotic shield around the procoagulant anionic phospholipids on the trophoblast surface, precluding the phospholipid-dependent coagulation reactions [17].

Since ANXA5 has a distinct role in maintaining blood fluidity in the placental circulation, it also presents an attractive candidate protein, linking placental haemostatic malfunction to the pathophysiology of PE. The aim of the present study was to investigate the possible role of ANXA5 in PE by comparing mRNA and protein levels in placentas derived from pregnancies complicated by PE and those from uneventful pregnancies.

**Materials and Methods**

**Sample Collection**

This study was approved by the Research and Ethics Committee of the University Hospital of Heraklion, Crete, Greece. Informed consent was obtained from all participants. Placentas were obtained after vaginal deliveries or caesarean sections from 23 women with singleton pregnancies that where complicated with PE and from 34 uncomplicated pregnancies. Biopsy specimens of the medial part of the maternal-fetal interface were obtained from each placenta, in such a way that each sample contained the decidua basalis and villous placenta, but not the chorionic plate [18,19]. Areas involving gross calcifications or infarcts were avoided. Contamination from fetal membranes was also minimized. Tissue biopsies were snap-frozen and stored at −80 °C until use. The rest of the placenta was sent for routine histological examination.

**Clinical Definitions and Sample Description**

Preeclampsia was defined as hypertension in previously normotensive women after 20 weeks of gestation (systolic blood pressure \(\geq 140\) mmHg or diastolic blood pressure \(\geq 90\) mmHg) on at least two occasions associated with proteinuria (\(\geq 300\) mg in a 24-hour urine collection or one dipstick measurement \(\geq 2+\)) [1]. Control group included women with uncomplicated, normotensive singleton pregnancies that delivered healthy, appropriate-for-gestational-age babies. Exclusion criteria were stillbirth, multiple gestations, chorioamnionitis, pre-pregnancy hypertension, renal disease, as well as chromosomal abnormalities and fetal anatomical defects. Baseline demographic characteristics and fetal anatomical defects. Baseline demographic characteristics and medical history information (maternal weight, height, age, parity, smoking, mode of delivery, fetal gender and birth weight) were recorded for all participants (Table 1).

**RNA Extraction and cDNA Synthesis**

RNA was extracted using the TRIzol\textsuperscript{®} reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol. RNA concentration and purity were measured on a NanoDrop 1000 spectrophotometer (NanoDrop Products, Wilmington, DE, USA).

cDNA was synthesized with the Thermoscript\textsuperscript{™} RT kit (Invitrogen), following the manufacturer's instructions, using random hexamers as amplification primers. cDNA was stored at −20 °C until use.

**Quantitative Real-Time Polymerase Chain Reaction (qPCR) Assay**

ANXA5 (Forward primer: 5'-CTTGGGCACAGATAGGAGAGCA-3'; Reverse primer: 5'-AAGCCGAGGTTTTCATCAGAC-3'; Amplicon size: 182 bp) mRNA expression was measured using a qPCR assay with SYBR\textsuperscript{®} Green dye, with β-Actin (Forward primer: 5'-CGGCACTGC

**Protein Extraction and Western Blot (WB)**

Proteins were extracted with the T-PER\textsuperscript{®} Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's protocol, and were stored at −80 °C until use.

30 μg of each protein specimen were separated by 12.5% SDS-polyacrylamide gel electrophoresis and were transferred to 0.45 μm nitrocellulose membranes (Thermo Scientific). Membranes were incubated with 1 μg/ml of mouse anti-ANXA5 (36 kDa) monoclonal antibody VAA-33: sc-65391 (Santa Cruz Biotechnology, Dallas, TX, USA) or mouse anti-Actin (43 kDa) antibody MAB1501 (Millipore, Billerica, MA, USA). After applying the AP124P goat anti-mouse peroxidase conjugated secondary antibody (Millipore), immunodetection was performed with the SuperSignal\textsuperscript{®} West Pico Chemiluminescent Substrate (Thermo Scientific), detected on Super RX X-ray films (Fujifilm, Tokyo.

**Table 1**

<table>
<thead>
<tr>
<th>Clinical characteristics of the study groups.</th>
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<tbody>
<tr>
<td>Preeclampsia pregnancies</td>
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<tr>
<td>Cases (n)</td>
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<tr>
<td>Maternal age (mean ± SD, years)</td>
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<td>BMI (mean ± SD)</td>
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<td>Maternal weight gain (mean ± SD, Kg)</td>
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<td>Gestational age at delivery (mean ± SD, wks)</td>
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<td>Birth weight (mean ± SD, gr)</td>
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<td>Mode of delivery</td>
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<td>Cesarean section (%)</td>
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<td>Parity</td>
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<td>Nulliparous (%)</td>
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<td>Multiparous (%)</td>
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<tr>
<td>Child gender</td>
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<td>Male (%)</td>
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<td>Smoking</td>
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<td>Yes (%)</td>
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<tr>
<td>No (%)</td>
</tr>
<tr>
<td>SGA Babies</td>
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<td>Yes (%)</td>
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<td>No (%)</td>
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SD: Standard Deviation; SGA: Small for Gestational Age.

* Student's T test (2-tailed);

b Chi-square or Fisher's exact test (2-tailed).
Japan). Protein expression was quantified using the Photoshop CS2 image analysis software (Adobe Systems, San Jose, CA, USA).

**Histology – Immunohistochemistry (IHC)**

The whole placenta was examined grossly and then sampled for routine microscopic examination with Hematoxylin-Eosin staining. Subsequently, 4 μm sections of selected paraffin-embedded tissue containing the basal plate and placental parenchyma were stained with the mouse anti-ANXA5 monoclonal antibody VAA-33: sc-65391 (Santa Cruz) diluted 1:50 in bovine serum albumin/Tris-buffered saline (BSA/TBS), pretreated in citrate buffer (pH 6.0). Secondary antibody and visualization were applied by the Envision Detection System (DAKO, Glostrup, Denmark).

**ANXA5 Scoring and Assessment of Labeling Index (LI)**

Immunohistochemical staining was assessed using one histological section per case (measuring 2 × 1 cm in average). Any staining intensity above background of immunolabeled cells was considered positive ANXA5 expression. For LI determination the SigmaScan Pro 5.0 software was used (Systat Software, Chicago, IL, USA). A percentage positive score was assessed in reference to the total area of immunolabeled cells within the placental parenchyma included in the section.

**Statistical analysis**

Pearson’s correlation or the non-parametric Spearman’s rho test was used to examine the association of ANXA5 mRNA and protein levels with continuous variables [maternal age, body mass index (BMI), weight gain, gestational age at delivery and birth weight centile]. Moreover, their association with categorical data (smoking habits, mode of delivery, child gender and parity) was examined using Student’s t test, or the nonparametric Mann–Whitney U- and Kruskal–Wallis H-tests. Additionally, the chi-square ($\chi^2$) or Fisher’s exact tests were used to examine ANXA5 expression levels with the various clinicopathological parameters after stratification. Statistical analyses were two-sided, and were performed with SPSS 11.5 (SPSS, Chicago, IL, USA). Statistical significance was set at the 95% level ($P < 0.05$).

**Results**

**Clinical Data analysis**

Women with PE gained less weight during their pregnancies ($p = 0.007$), gestation was 5 weeks shorter ($p < 0.001$) and birth weight was at least 1 Kg lower ($p < 0.001$). Additionally, they were more obese ($p < 0.004$), gave birth with a caesarian section ($p < 0.001$) and to smaller babies ($p < 0.001$) (Table 1).

**Real-Time PCR analysis**

qPCR analysis revealed that the average normalized ANXA5 mRNA expression in PE samples was less than 1% of its expression in normal samples (mean ± SD: 0.002 ± 0.004 vs. 0.55 ± 0.38, $p < 0.001$), with 22/23 (96%) of samples being downregulated and only 1/23 (4%) having normal mRNA expression (Fig. 1).

**Western Blot analysis**

Western Blot analysis only partially verified the above results, since ANXA5 protein was downregulated in 13/23 (57%) PE samples when compared with normal specimens, with expression levels approximately at 65% of the average normal expression (mean ± SD: 0.53 ± 0.30 vs. 0.81 ± 0.25, $p = 0.001$) (Fig. 2A, 2B).

Statistical analysis of the WB data revealed that ANXA5 protein expression was increased in PE women that smoked vs. PE women that were not smokers (mean ± SD: 0.64 ± 0.23 vs. 0.41 ± 0.33, $p = 0.027$) (Fig. 2C).

**Histology – Immunohistochemistry**

Uteroplacental vascular disorders were the main histopathological findings in all PE samples, including macroscopic and microscopic basilar infarcts, retroplacental hematoma, decidual vasculopathy, as well as areas with histological indications of maternal hypoperfusion (e.g. increased syncytial knots, reduced vasculosyncytial membranes, distal villous hypoplasia or villous agglutination). Subacute or chronic lesions of fetal vessel thrombosis were observed in 6/23 (26.1%) PE placentas.

ANXA5 immunohistochemical expression was observed in the perivillous and extravillous trophoblast of all examined placentas. The staining pattern was heterogeneous with a tendency of ANXA5 positive cells towards cluster formation at the sites of syncytiot knots. Immunostaining was mostly visualized as a continuous line along the apical site...
of the perivillous syncytiotrophoblastic cells or was localized in the cytoplasm of isolated trophoblastic cells (Fig. 3A). Extravillous trophoblastic cells also showed perinuclear localization of immunostaining. The expression pattern was granular, consisting of fine dusty and coarse granules (Fig. 3A). As a rule, in both PE and control placentas, staining intensity was increased within clusters of syncytial knots, as well as in areas of villous adhesion, as seen in hypoperfused or freshly infarcted areas (Fig. 3B). Clusters of nonviable trophoblastic cells entrapped within old infarcts were invariably positive (Fig. 3D).

PE placentas and controls were classified in 3 categories according to the labelling index (LI = Percentage × Staining Intensity); those with high (>66%), medium (33-66%) and low (<33%) LI. PE placentas had lower LIs (22% high; 11% medium; 67% low) than controls (80% high; 20% medium) (p = 0.020). In addition, control placentas presented a diffuse pattern of staining along the perivillous syncytiotrophoblast lining (Fig. 3B). In contrast, PE placentas showed a focal staining pattern, accentuated within clusters of syncytial knots in the context of the Tenney-Parker phenomenon (Fig. 3C, 3D); diffuse perivillous lining was prominent only in areas of hypoperfusion indicated by villous clustering and adhesion.

Discussion

The hypothesis that we investigated in the present study was, whether placental dysfunction associated with PE correlates with altered ANXA5 mRNA and protein expression. We found decreased ANXA5 mRNA and protein levels in placental tissues derived from pre-eclamptic women compared to those with uncomplicated pregnancies. Immunohistochemistry confirmed the reduced protein levels, since pre-eclamptic placentas demonstrated low staining intensities for ANXA5.

ANXA5 is present on the apical surface of the perivillous trophoblasts facing circulating maternal blood. As a result of its localization, it is capable of promoting the fluidity of maternal blood circulating through the intervillous space [20], thus ensuring materno-fetal nutrition exchange through diffusion [21]. The majority of PE samples exhibited significantly lower ANXA5 mRNA levels compared to controls. Reduced ANXA5 mRNA has been also reported in previous studies that assessed placental expression of ANXA5 in women with PE, and has been correlated with disease onset and the presence of FGR [22–24]. Protein levels in maternal blood were also reduced in a similar study [23]. Graded immunohistochemical expression confirmed these findings and correlated the reduced protein expression with the severity of PE [25]. However, this finding has not been reproduced by all researchers, since Ormahi et al. found ANXA5 expression to be related only to FGR but not to PE or its clinical severity [22].

Western Blot analysis demonstrated that placentas from the PE group also exhibited reduced protein levels (albeit only partially). According to the thromboregulatory action of ANXA5, reduced protein expression would allow the binding of coagulation factors to phospholipid surfaces and therefore induce thrombosis. Placental vasculopathy and ischemic infarcts are associated with PE, particularly in cases of inherited thrombophilia and antiphospholipid syndrome (APS) [26,27]. The observed downregulation of ANXA5 transcript and protein levels probably contributes to thrombotic predisposition and thus to placental dysfunction.

An alternative explanation for the reduced ANXA5 levels might be an underlying genetic defect that predisposes to ANXA5 mRNA down-regulation. Bogdanova and co-workers reported a variation of four consecutive nucleotide substitutions in the ANXA5 promoter that might create a pathologically hypoxic environment [32,33]. We would speculate that the increased ANXA5 protein levels found in PE smokers indicate a possible implication in the procedure of decidual vessel remodelling, which underlies the pathogenesis of PE.
Noteworthy, there is a disassociation between ANXA5 mRNA levels, which are nearly abolished in PE, and protein expression levels, which are only moderately reduced. This discordance could be explained by factors that decrease mRNA longevity post-transcriptionally or increase protein stabilization and reduce degradation post-translationally. Additionally, ANXA5 levels are not universally upregulated or downregulated, at least in Systemic Lupus Erythematosus (SLE), in which ANXA5 levels are increased in peripheral blood mononuclear cells (PBMCs), while they are decreased in serum (26.8 ± 3.0 ng/ml for SLE patients versus 49.0 ± 3.3 ng/ml for controls) [34]. Although we lack data regarding ANXA5 levels/concentration in the serum of pregnant women complicated by PE, we could assume that reduced ANXA5 levels are a placental-related characteristic and not a systemic finding. This is supported by our IHC findings, in which ANXA5 is present in extravillous trophoblasts of decidual basalis, despite the reduced ANXA5 gene expression within intravillous cells. This finding should come as no surprise, since hypoperfusion-hypoxic cell compartments are expected to demonstrate higher ANXA5 function. Apart from the extravillous trophoblasts, it is also possible that ANXA5 protein could originate from the plasma pool and/or the circulating cells. There are other possible explanations regarding this discrepancy. Cell-surface ANXA5 molecules need negative phospholipids for binding [35]. Perhaps one of the known ANXA5 modifications (such as the R23E mutation) or a yet to be discovered alteration in the repartition of anionic phospholipids in the outer membrane of trophoblastic cells in PE, could result in reduced ANXA5 cell surface expression. Anti-ANXA5 antibodies could also play an important role, since they are present in about 20% of PE patients [36], as well as in SLE patients with thrombotic and thrombocytopenic complications [37]. Finally, it is well established that gonadotropin-releasing hormone (GnRH) and human chorionic gonadotropin (hCG) increase ANXA5 levels [38], while prolactin decreases them [39]. Prolactin interacts with trophoblastic cells, stimulating cell migration and invasion [40], while prolactin levels are very high in preeclamptic women [41]. It is possible that ANXA5 levels are reduced because a prolactin-induced ANXA5 antagonist is upregulated in the trophoblasts during PE.

In conclusion, we have demonstrated that the placental expression of ANXA5 is decreased in pregnancies complicated with PE. These results suggest that decreased ANXA5 expression and activity could be directly involved in the impaired mechanism of vascular adaptation and defective decidual vessels blood fluidity. Further studies are needed in order to verify our results and to investigate whether ANXA5 levels in the maternal circulation could be used as a biological marker for the early detection of preeclampsia, as well as for the prediction of its severity and outcome.

**Conflict Of Interest Statement**

The authors have no conflict of interest to declare.

**References**


