Complement inhibitory proteins expression in placentas of thrombophilic women

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Abstract: Factors controlling complement activation appear to exert a protective effect on pregnancy. This is particularly important in women with thrombophilia. The aim of this study was to determine the transcript and protein levels of complement decay-accelerating factor (DAF) and membrane cofactor protein (MCP) in the placentas of women with acquired and inherited thrombophilia. Also, we assessed immunohistochemistry staining of inhibitors of the complement cascade, DAF and MCP proteins, in the placentas of thrombophilic women. Placentas were collected from eight women with inherited thrombophilia and ten with acquired thrombophilia. The levels of DAF and MCP transcripts were evaluated by qPCR, the protein level was evaluated by Western blot. We observed a higher transcript (p < 0.05) and protein (p < 0.001) levels of DAF and MCP in the placentas of thrombophilic women than in the control group. DAF and MCP were localized on villous syncytiotrophoblast membranes, but the assessment of staining in all groups did not differ. The observed higher expression level of proteins that control activation of complement control proteins is only seemingly contradictory to the changes observed for example in the antiphospholipid syndrome. However, given the hitherto known biochemical changes associated with thrombophilia, a mechanism in which increased expression of DAF and MCP in the placentas is an effect of proinflammatory cytokines, which accompanies thrombophilia, is probable. (Folia Histochemica et Cytobiologica 2012, Vol. 50, No. 3, 460–467)

Key words: thrombophilia, complement, placenta, DAF, MCP

Introduction

Antiphospholipid Syndrome (APS) and coexisting thrombosis are being extensively studied as potential causes of pregnancy loss. The loss is more likely to occur in the first trimester of pregnancy as a result of impaired implantation, whereas intrauterine demise, placental abruption or preeclampsia are more frequent in later pregnancy [1]. Although the frequency of thrombophilia in pregnant women is lower than previously believed, the association between various types of antiphospholipid (aPL) antibodies, factor V Leiden and prothrombin mutation is an evident cause of pregnancy complications [2].

Despite numerous studies, the mechanism of pregnancy loss in women with APS remains unclear. Antiphospholipid antibodies (aPL) in vitro inhibit the anticoagulant function of annexin V, protein C and block fibrinolysis [3–6]. Besides activating the complement cascades, aPL (including β₂-glycoprotein1 antibodies (anti-β₂GPI) in the placenta) blocks the β₂GPI protein as a ligand for receptors on epithelial and trophoblast cells surface, resulting in decreased hCG production and impaired expression of protein on cell surface (integrins, catherins). Anti-β₂GPI binding to trophoblast blocks its invasion inducing apoptosis. Anti-β₂GPI has been observed to affect trophoblast function, decreasing the expression...
of the growth factor: heparin binding EGF-like factor (HB-EGF) [7].

Complement activation due to aPL induces inflammatory reaction through proinflammatory cytokines including TNFα, thus impairing the balance between Th1/Th2 which is favorable in pregnancy [8]. The inflammatory reaction is also present in thrombophilic women not related to APS. The key moment of this process is the activation of prothrombin into thrombin. Thrombin has a wide spectrum of action, fusing together proinflammatory and prothrombotic action and stimulating proinflammatory cytokine expression [9, 10].

Tissues including placenta possess the ability to inhibit complement cascade through the presentation of receptors for complement elements and membrane proteins degrading complement elements on the surface of cells. The complement decay-accelerating factor (DAF), also known as CD55, is among many well-studied complement control proteins. This membrane protein prevents the assembly of the C3bBb complex, thus blocking the formation of the membrane attack. Another well-known complement inhibitory factor is the membrane cofactor protein (MCP), also known as CD46. MCP protects the host cells from damage as cofactor for inactivation of complement components C3b and C4b by serum factor I.

It has been demonstrated that murine homoygotes without the Crry (Crry-/-) gene, encoding protein of analog action to human DAF and MCP, experience fetal absorption or growth impairment [11, 12].

The role of the complement controlling cascade factors in thrombophilia is poorly understood. Rai et al. (in 2006) showed that in non-conception cycle, the level of mRNA for DAF in the endometrium of APS patients is statistically lower compared to healthy women [13]. We can speculate that activation of complement cascade in APS may be associated with weakened expression of complement cascade inhibitors. Secondary complement activation takes place also in inherited thrombophilia through thrombin activation [10]. To date there have been very few papers concerning molecular changes in the placentas of women with thrombophilia. Nor is the status of complement controlling factors in thrombophilic patients well understood.

The aim of this study was to investigate whether in the placentas of thrombophilic women there is an altered expression of DAF and MCP proteins, and whether differences in the expression of these proteins are dependent on the form of thrombophilia.

Material and methods

Material. The study was conducted at the Division of Reproduction, Department of Obstetrics and Gynecology, Karol Marcinkowski Poznan University of Medical Sciences, Poland, in 2011.

We analyzed 19 placentas from 18 patients with inherited (n = 8) and acquired (n = 10) thrombophilia; 20 placentas from uneventful pregnancies which resulted in the delivery of a healthy infant constituted the control group (n = 20).

Patients with APS fulfilled at least one clinical and one laboratory criterion. Clinical criteria included: (1) three or more early miscarriages; or (2) two consecutive late miscarriages (beyond 12 weeks of gestation); or (3) intrauterine fetal death beyond ten weeks of gestation of unknown cause or delivery before 34 gestational weeks complicated by preeclampsia with placental insufficiency; or (4) thrombotic episodes.

Laboratory criteria included: (1) presence of lupus anticoagulant (LA); (2) moderate to high titers (≥ 20 GPL or MPL) of anticardiolipin antibodies (aCL) or substantial titers (> 99 percentile) of anti-β2GP1. In molecular tests, eight women were diagnosed with inherited thrombophilia: factor V Leiden was found in five, hyperhomocysteinemia in two, and protein S deficiency in one patient. All pregnant patients diagnosed with thrombophilia were treated with either prophylactic or therapeutic doses of LMWH and/or aspirin, depending on their weight and thrombotic episodes.

The clinical characteristics of the studied groups are shown in Table 1.

The study protocol was approved by the local ethical committee and the patients signed an informed consent form. All coagulation tests were performed at the Hemostasis Laboratory of Medical University Hematology Department in Poznan. The laboratory has a TUV Rheinland ISO9001 certificate.

Method. Directly after birth, a small part of the placenta was placed in RNAlater and frozen at minus 20°C, for the assessment of transcript levels. Another part was frozen in liquid nitrogen (LN) until Western blot assessment was performed. The specimens included both maternal and fetal fragments, but the decidual and amniochorional membranes were removed. The entire placenta was fixed in buffered formalin for histopathological assessment and DAF and MCP immunolocalization.

RNA isolation and Real-time quantitative PCR (RQ-PCR) analysis of DAF and MCP transcript levels. Total RNA was isolated from 30 mg of placenta by RNeasy Protect Mini Kit Qiagen (Hilden, Germany). RNA samples were treated with DNase I from DNase Set Qiagen (Hilden, Germany), quantified, and 1 μg reverse-transcribed into cDNA, using QuantiTect Reverse Transcription Kit and random hexamer primers from Qiagen (Hilden, Germany). RQ-PCR was conducted in a Corbett Research Rotor-Gene 3000 thermocycler (Mortlake, Australia). Target cDNA was quantified using the relative quantification method employing a calibrator. The calibrator was prepared as a cDNA mix from all patients and control samples and consecutive dilutions were used to create a standard curve as provided in the
### Table 1. Clinical characteristics of women with thrombophilia and control group

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Inherited thrombophilia (n = 8)</th>
<th>Acquired thrombophilia (n = 10)</th>
<th>Control group (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal characteristics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal age (mean ± SD)</td>
<td>30.2 ± 3.16</td>
<td>31.4 ± 3.04</td>
<td>29.7 ± 3.97</td>
</tr>
<tr>
<td>Gravity (mean ± SD)</td>
<td>1.75 ± 1.39</td>
<td>2.18 ± 1.6</td>
<td>1.1 ± 0.31</td>
</tr>
<tr>
<td>Parity (mean ± SD)</td>
<td>0.38 ± 0.52</td>
<td>0.55 ± 0.93</td>
<td>1.1 ± 0.31</td>
</tr>
<tr>
<td>History of pregnancy failures*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early miscarriages</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Late miscarriages</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>IUD</td>
<td>3</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Previous thrombotic episodes</td>
<td>2</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Laboratory criteria**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aCL</td>
<td>–</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>anti-β2GPI</td>
<td>–</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>LA</td>
<td>–</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>F V Leiden</td>
<td>5</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Hyperhomocysteinemia</td>
<td>2</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Protein S deficiency</td>
<td>1</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Characteristics of current pregnancy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placental weight [g]</td>
<td>562.86 ± 164.39</td>
<td>509.55 ± 226.54</td>
<td>530.0 ± 96.57</td>
</tr>
<tr>
<td>Fetal weight [g]</td>
<td>2,893.75 ± 1,285.22</td>
<td>2,578.33 ± 1,269.47</td>
<td>3,351.0 ± 398.15</td>
</tr>
<tr>
<td>Gestation age (wk)</td>
<td>36.75 ± 3.99</td>
<td>34.91 ± 6.35</td>
<td>39.2 ± 1.32</td>
</tr>
<tr>
<td>Vaginal delivery</td>
<td>3</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Cesarean section</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

*A number of women with thrombophilia showed more than one clinical criterion eligible for laboratory diagnostics;  
**In two patients with acquired thrombophilia, we found concurrence of aCL and LA; in one concurrence of aCL, LA, and anti-β2GPI

### Table 2. Sequence of primers used for qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5'-3' direction)*</th>
<th>Position</th>
<th>ENST number</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAF</td>
<td>GGTGCAACCATCTCTTTCTC TGG TGG TGC TGG ACA ATA AA</td>
<td>+805; +825 +926; +946</td>
<td>00000196352</td>
<td>141</td>
</tr>
<tr>
<td>MCP</td>
<td>TGTATTGTACCAAGGGATGGA AACTGCTTGGCTAGGGACTC</td>
<td>+2,537; +2,558 +2,615; +2,636</td>
<td>00000117335</td>
<td>99</td>
</tr>
<tr>
<td>ACTB</td>
<td>GCACCACACCTCTCACAATGACG GGTAGCACAGCTGGCTAGGACAC</td>
<td>+450; +473 +592; +616</td>
<td>00000331789</td>
<td>166</td>
</tr>
</tbody>
</table>


Relative Quantification Manual from Roche Diagnostics GmbH (Mannheim, Germany).

For amplification, 60 ng of total cDNA solution was added to 20 μl (total) of DyNAmo HS SYBR* Green qPCR Kit from Finnzymes (Espoo, Finland) and primers (Table 2). One RNA sample of each preparation was processed without the reverse transcription (RT)-reaction to provide a negative control in subsequent PCR. The quantity of investigated transcripts in each sample was standardized by human β-actin (ACTB) transcript levels. The DAF and MCP mRNA levels were expressed as the multiplicity of these cDNA concentrations in the calibrator.

**Protein isolation and Western blot analysis.** The crushed in LN material was mixed with SB buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoetha-
nol) and total protein concentration was estimated using a commercial 2-D Quant kit (GE Healthcare). Samples containing 30 μg of protein were separated by electrophoresis in a 4–12% SDS-polyacrylamide gel (Bis-Tris NuPAGE, Invitrogen). The separated proteins were transferred to a PVDF membrane and blocked with TBST (TBS plus 0.1% Tween-20) containing 4% BSA.

Immunodetection was processed with the use of rabbit polyclonal antibody anti-DAF (H-319, 200 μg/ml) and rabbit polyclonal antibody anti-MCP (H-294; 200 μg/ml) in 1:200 concentrations (SantaCruz, USA). For ACTB detection, rabbit polyclonal anti-ACTB antibody was used in a 1:400 concentration (N-21; 100 μg/ml), (SantaCruz, USA). Goat anti-rabbit, conjugated with Alexa Fluor 663 (Invitrogen, USA), was used as the secondary antibody. Bands were revealed using the Fuji FLA5100 (FujiFilm, Japan) scanner. The quantities of Western blot-detected DAF, MCP and β-actin proteins were determined based on the band optical density. The band densitometry readings were normalized to ACTB loading control to calculate the studied protein to β-actin optical density ratio. As reference for statistical calculations, the mean value of DAF and MCP in the control group was determined as 1.00.

Immunohistochemistry. Samples from the placenta in unchanged regions were taken for immunohistochemistry. Specimens were preserved in buffered formalin (pH 7.4–7.6), immersed in paraffin blocks and dissected into pieces up to 3–4 μm thick. A part of the sections was stained with hematoxylin and eosin (H + E) and neighboring sections underwent immunohistochemical staining to identify the presence of DAF and MCP antigen. Retrieval of antigens was processed in Retrieval Solution Buffer (DAKO, Denmark), 98.5°C. To detect DAF and MCP, the same primary antibodies were used as in WB analysis in 1:100 dilution. Reaction with secondary antibodies and staining were performed with Dako REAL™EnVision™ Detection System Peroxidase/DAB, (DAKO, Denmark). With every series of staining, we performed a negative control without primary antibody.

Assessment of the immunohistochemical reaction was conducted by a pathologist in random order, with no knowledge of the study group affinity. The expression of DAF and MCP proteins was assessed with the use of a 0–3 point scale (0 — no reaction (Figure 1A); 1 — weak reaction, discontinuous in both membranes in over 50% of the villi (Figure 1B); 2 — distinct reaction, continuous in one of the membranes of syncytiotrophoblast: superficial (arrow) and basal (dual arrow) (D))

Figure 1. Immunohistochemical staining for DAF and MCP proteins, magnification × 400. Lack of reaction (A). Subtle reaction in the outer membrane of syncytiotrophoblast (arrow) (B). Continuous reaction in the outer membrane of syncytiotrophoblast (arrow); discontinuous in the basement membrane (dual arrow) (C). Continuous reaction in both membranes of syncytiotrophoblast: superficial (arrow) and basal (dual arrow) (D)
membranes in more than 50% of the villi (Figure 1C); 3 — distinct reaction in both membranes in more than 50% of the villi (Figure 1D).

**Statistical analysis.** For statistical analysis, SigmaStat3.5 software was used. The analysis of the results was based on the Mann–Whitney Rank Sum test and Chi Square test for staining assessment. p < 0.05 was considered statistically significant.

**Results**

Levels of DAF and MCP transcript and protein in placentas of women with thrombophilia and controls

We used RQ-PCR and Western blot analysis to evaluate DAF and MCP transcript and protein levels, respectively, in the placentas of women diagnosed with thrombophilia and the controls. In women with acquired thrombophilia, we observed significantly higher levels (p < 0.05) of DAF and MCP transcript in placentas with APS group compared to controls: 2.2 times for DAF and 2.29 times for MCP. In placentas from women with inherited thrombophilia, we also observed significantly higher levels (p < 0.05) of DAF and MCP transcript in placentas from the thrombosis group compared to controls: 4.52 times for DAF and 3.74 times for MCP (Table 3, Figures 2A, B).

We also found significantly higher levels of DAF and MCP protein (p < 0.001) in placentas with acquired thrombophilia compared to controls: 3.26 times for DAF and 3.83 times for MCP. In the inherited thrombophilia group, we found 3.08 times higher level of DAF protein and 3.94 times higher level of MCP protein (p < 0.001) (Table 3, Figures 3A, B). Additionally, we found no difference in transcripts and proteins DAF and MCP between the inherited and acquired thrombophilia groups (data not shown).

**Immunohistochemistry staining**

Generally, a strong color reaction in the chorian syncytiotrophoblast membranes was observed for both tested proteins. Statistical analysis showed no statistically significant differences in the scale of staining for MCP and DAF. The results of histological evaluation of microscopy slides, stained for the presence of proteins DAF and MCP, are shown in Table 4. In samples of the controls, stained with the anti-DAF antibody, a higher (although not statistically significant) percentage of staining assessed as 3 points, and a higher percentage of staining evaluated at 0 and 1 in the group of placentas from thrombophilic women, is worth noting.

![Table 3. Relative transcript and protein levels of DAF and MCP in placentas from women with thrombophilia and control group](https://www.fhc.viamedica.pl)
Discussion

Expression of complement activation proteins including MCP and DAF in trophoblast tissue is already observed in the first trimester of pregnancy. A strong expression of these proteins on trophoblast surface activates the mechanisms protecting the fetus from maternal immunological response [14, 15]. Hill et al. (1995) did not find any differences in DAF and MCP expression in the placentas of women with recurrent pregnancy losses in the first trimester compared to healthy fertile women [16]. Rai et al. (2006) proved a decreased transcript level of DAF in the endometrium of women with "

Table 4. Assessment of immunohistochemical staining for DAF and MCP proteins. The table shows the number of patients presenting successive staining steps (0–3) of tissues for the presence of DAF and MCP

<table>
<thead>
<tr>
<th>Degree of immunohistochemical assessment</th>
<th>DAF</th>
<th>MCP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Acquired thrombophilia (%)</td>
<td>0</td>
<td>2 (18%)</td>
</tr>
<tr>
<td>Inherited thrombophilia (%)</td>
<td>1 (12.5%)</td>
<td>3 (37.5%)</td>
</tr>
<tr>
<td>Control group (%)</td>
<td>0</td>
<td>1 (5%)</td>
</tr>
</tbody>
</table>

*Chi², p = 0.099; *Chi², p = 0.201
APS [13]. There is no data regarding the expression of complement activating proteins in mature placentas of thrombophilic women. Our results are in contrast with common scheme, where insufficient placental protection from DAF favors C3 convertase activation, resulting in inflammatory reaction in the placenta.

We observed significantly increased mRNA and protein expression of DAF and MCP in the placentas of women either with inherited thrombophilia or related to APS. It seems less likely that high expression of factors controlling the initial stage of complement cascade in APS women is primary. Otherwise we would have never observed the aPL related proinflammatory effect. aPL inhibiting the expression of HB-EGF would give an opposite effect to the one we found. The relationship between HB-EGF expression and DAF and MCP [17, 18] has been proved in both in vitro and in vivo studies.

An inhibiting function of aPL on HB-EGF secretion was confirmed in vitro only for the anti-\(\beta\),GP1 antibody. Simultaneously, it was confirmed that LMWH inhibits reaction of anti-\(\beta\),GP1 with trophoblast [10].The influence of other types of antibodies on HB-EGF expression has not been determined.

In our study, in the group of women with APS, only one woman had an elevated titer of anti-\(\beta\),GP1. We cannot exclude the possibility that the high expression of DAF and MCP in women with APS is indirectly a result of the treatment with therapeutic doses of LMWH or aspirin. In our study we found a statistically higher expression of DAF and MCP in both variants of thrombophilia.

There is no convincing data that could explain a primary high level of DAF and MCP in the placentas of women with inherited thrombophilia, where the inflammatory reaction mediator is likely the thrombin [10, 19]. LMWH mechanism of action known from APS related thrombophilia is not applicable there. Considering the lack of difference in DAF and MCP level in both types of thrombophilia, it is less likely that LMWH has a stimulating effect on HB-EGF expression and indirectly on DAF and MCP.

Mason et al. (1999) observed a stimulating influence of TNF \(\alpha\) in vitro on DAF expression in endothelial cells. There was no analog reaction as far as MCP was concerned [20]. Increased DAF expression found in our studies can be explained as a secondary response of placental tissue to inflammatory reaction and the presence of proinflammatory cytokine that can activate complement cascade in a secondary way [9, 10, 20]. Lack of similar observations in MCP in the referenced literature might be a result of the limited possibilities of an in vitro model. It is very likely that a similar mechanism of secondary response to inflammation is associated with MCP expression in vivo. We support the theory that the DAF and MCP expression, as a secondary reaction to proinflammatory factors, is more likely. The presence of proinflammatory cytokines and thrombin is common for both variants of thrombophilia. Further studies are needed in order to explain this phenomenon.

Considering the limitation of quantitative analysis of protein expression in specimens stained immunohistochemically, only qualitative differences in placental distribution DAF and MCP were analyzed. There were no statistically significant differences in the evaluation of distribution of both proteins in the placenta, indicating no differences in cells mechanism responsible for the expression of analyzed placental factors.

To the best of our knowledge, this is the first publication analyzing the expression of DAF and MCP in the placentas of thrombophilic women. In this study we did not estimate the cytokine level. However, taking into consideration the abovementioned publication results of Mason et al., we can expect an elevated level of proinflammatory cytokines in the placentas of the thrombophilic group. Further studies are needed to elucidate whether proinflammatory cytokines in the placenta influence the expression of DAF and MCP expression and, if they do, what spectrum should be taken into consideration.

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References


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