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Placental Dysferlin Expression is Reduced in Severe Preeclampsia

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Abstract

Dysferlin (DYSF) and myoferlin (MYOF), members of the ferlin family of membrane proteins, are co-expressed in human placental syncytiotrophoblast (STB). Although the role of these ferlin proteins in the placenta has yet to be established, it has been suggested that DYSF and MYOF may contribute to the stability of the apical STB plasma membrane. The release of STB-derived cellular debris increases in the setting of preeclampsia (PE), suggesting relative destabilization of the hemochorial interface. To test whether PE was associated with alterations in placental expression of DYSF and/or MYOF, a cross-sectional study was performed using specimens of villous placenta collected from women with severe PE (n = 10) and normotensive controls (n = 10). DYSF and MYOF expression were examined using quantitative real-time RT-PCR, immunoblotting, and immunofluorescence labeling of tissue specimens. Placental DYSF expression was 57% lower at the mRNA level (p = 0.03) and 38% lower at the protein level (p = 0.026) in severe PE as compared to normotensive subjects. There were no differences in placental MYOF protein or mRNA expression between these groups. No appreciable changes in the distribution of DYSF or MYOF within placental villi were observed in PE relative to control specimens. We conclude that DYSF expression is reduced in severe PE relative to gestational age-matched controls. As DYSF has a role in membrane repair, these data suggest a role for DYSF in the stability of the apical STB plasma membrane and may account, at least in part, for the increased shedding of microparticles from this membrane in PE.

Keywords

dysferlin; myoferlin; preeclampsia; placenta

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Introduction

Throughout gestation, the human placenta undergoes an impressive amount of membrane damage and repair. As part of routine turnover, sprout-like structures containing clusters of densely heterochromatic nuclei and cellular debris (commonly referred to as syncytial knots or sprouts, although controversy exists with regard to the specificity of these terms [1]) arise from the apical syncytiotrophoblast (STB) and are shed into the maternal circulation [2–4]. It has been estimated that the average term placenta sheds between 1.5×10^5 [5] and 8.5×10^5 [6] STB-derived particles daily. How the apical plasma membrane of the STB repairs itself after syncytial deportation remains unknown, although one assumes the placenta has developed a highly efficient and rapid mechanism for local membrane repair. Indeed, such repair must occur or else damage to the STB would be extensive.

While the mechanisms that contribute to the repair of damaged plasma membranes have not been completely explicated, current consensus suggests that spontaneous resealing (excepting special circumstances) is unlikely [7]. As such, all cells and tissues are believed to require system(s) whereby foci of damaged membranes can be rapidly contained. One widely endorsed hypothesis holds that plasma membrane disruptions are repaired by “patches” arising from intracellular membrane vesicles [7]. In most instances, this is believed to occur through Ca^{2+} -regulated exocytotic fusion of endomembranous material with the damaged membrane bilayer [7;8].

Dysferlin (DYSF) and myoferlin (MYOF) are two proteins of the ferlin family that contribute to the repair of damaged plasma membranes in striated muscle [7;9–11]. Six ferlin paralogs with sequence homology with the *Caenorhabditis elegans* FER-1 protein have been described in mammalian systems. In addition to DYSF (also known as FER1L1) and MYOF (also known as FER1L3), members include otoferlin (OTOF, also known as FER1L2), FER1L4, FER1L5, and FER1L6. Ferlin family proteins appear to share conserved, Ca^{2+} -responsive mechanisms whereby membrane fusion events are regulated [12]. The importance of ferlin-dependent membrane repair is highlighted by the pathobiological phenotypes that arise when one of these proteins fails in its normal function. In *C. elegans* and *Drosophila melanogaster*, ferlin mutations lead to impaired spermatogenesis and infertility [13–15]. In mice and humans, mutations in DYSF result in certain muscular dystrophies and cardiomyopathy [11;16–19], while OTOF deficiency produces sensorineural deafness [20;21]. Given that DYSF and MYOF are co-expressed in placental trophoblast [22;23], it has been speculated that these proteins might also contribute to STB plasma membrane repair [24].

Preeclampsia is associated with an increase in the amount of trophoblast-derived cellular debris within the maternal circulation [25–27]. Microparticles derived from STB shedding, together with other vasculopathic factors, are thought to contribute to the systemic manifestations of preeclampsia [28]. Preeclamptic placentas are characterized histologically by advanced villous maturation and an increase in the density of syncytial knots [29;30]. Such placentas also contain abnormalities at the ultrastructural level, including focal syncytial necrosis and loss or distortion of STB microvilli [3;31;32]. These pathological features are likely to represent events both antecedent and subsequent to trophoblast deportation [6;33], and suggest that preeclampsia is associated with destabilization of the STB plasma membrane. We have speculated that disturbances in the concentration or function of DYSF and/or MYOF might contribute to STB plasma membrane instability in the setting of preeclampsia [24]. The current study was designed to address whether severe preeclampsia is associated with changes in the expression and/or distribution of placental DYSF and MYOF.

Methods

Reagents and Supplies

A mouse monoclonal antibody against DYSF (clone Ham1/7B6) was purchased from Vector Laboratories (Burlingame, CA). A mouse polyclonal antibody against MYOF was obtained from Novus Biologicals (Littleton, CO). A rabbit polyclonal antibody against MYOF was obtained from Sigma-Aldrich (St. Louis, MO). Mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Chemicon International (Temecula, CA). TaqMan Universal Master Mix and Assays-on-Demand gene expression target assay mixtures for the detection of dysferlin (Hs00243339_m1), myoferlin (Hs01053408_m1), and the RPLPO large ribosomal protein (4310879E) were obtained from Applied Biosystems (Foster City, CA).

Precast Precise 10% polyacrylamide gels and SuperSignal chemiluminescent detection reagents were obtained from Thermo Scientific (Rockford, IL). ProLong antifade mounting reagent and Alexa Fluor-conjugated secondary antibodies were purchased from Molecular Probes-Invitrogen (Eugene, OR). TRIzol reagent and the SuperScript III first-strand synthesis system for RT-PCR were from Invitrogen (Carlsbad, CA). RNeasy mini columns and the RNase-Free DNase Kits were purchased from QIAGEN (Valencia, CA). All other reagents unless otherwise specified were obtained from Sigma-Aldrich (St. Louis, MO).

Tissue Collection

A cross-sectional study was performed using human placental specimens obtained with informed consent according to a protocol approved by the Biomedical Sciences Institutional Review Board at Ohio State University, Columbus, OH. Eligible subjects included English-speaking women 18–45 years of age delivered of a live singleton infant between 20 and 42 weeks of gestation at The Ohio State University Medical Center. Subjects with major structural fetal abnormalities or prenatally diagnosed aneuploidy were excluded. Given that the current study was exploratory in nature, we sought to examine the expression of placental ferlins among the more extreme cases of preeclampsia (PE) relative to controls, with the expectation that any significant differences could be explored in greater detail in subsequent studies. To this end, cases were restricted to women diagnosed with severe preeclampsia (PE), as established by the National High Blood Pressure Education Program Working Group Report on High Blood Pressure in Pregnancy [34]. For each case, a normotensive control subject matched for gestational age at delivery (± 7 days) was recruited. The criterion of matching for gestational age was instated based on prior data, in which DYSF protein expression was found to be approximately three times higher in first-trimester relative to term placental specimens [22].

Three representative biopsies of villous placental tissue, free of evident pathology, were collected from the maternal surface of each placenta by dissecting ~2-cm sections between the basal and chorionic surfaces. In all cases, tissue was processed as soon as possible following delivery of the placenta (within 10 min). Placental tissue was either fixed in 4% paraformaldehyde (PFA) or thoroughly washed in PBS and flash frozen in liquid nitrogen and stored at -80°C until used.

Total RNA Extraction and Real-Time RT-PCR

For total RNA extraction from frozen tissues, samples (~60–120 mg) were ground with a mortar and pestle under liquid N_2 and immediately placed in TRIzol reagent (Invitrogen). Total RNA was extracted according to the manufacturer's instructions, with modification. Briefly, following chloroform extraction and centrifugation, the resulting aqueous phase was applied to an RNeasy mini column (QIAGEN) and processed according to the manufacturer's protocol. This included on-column DNase digestion using the RNase-Free DNase kit (QIAGEN) to

remove contaminating genomic DNA. RNA was quantified by spectrophotometric absorbance at 260 nm, and 2 µg from each sample was then reverse-transcribed to cDNA using oligo dT primers (Invitrogen) and SuperScript III reverse transcriptase (Invitrogen).

For quantitative real-time RT-PCR, cDNA was amplified with Assays-on-Demand gene expression target assay primer/probe mixtures for DYSF and MYOF, and detected with a Prism 7300 sequence detector (Applied Biosystems). Amplification mixtures contained 2.5 µl of the appropriate target assay mixture, 1 µl of first-strand cDNA synthesis mixture (corresponding to 50 ng of reverse-transcribed RNA), 25 µl of TaqMan Universal Master Mix (Applied Biosystems), and nuclease-free distilled, deionized water to a total volume of 50 µl. Amplification was performed over 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min.

Amplification curves were analyzed using software provided by the manufacturer (Applied Biosystems). For each target gene, duplicate amplifications were performed. The technician performing the assay was blinded to the status (severe PE vs. control) of each sample. Using cycle threshold (C_T) values, target gene expression was normalized to the expression of RPLPO mRNA (endogenous control), as determined in parallel reactions. The choice of RPLPO as an appropriate endogenous control in villous placental tissue was based on both our prior experience (unpublished observations) as well as the work of others [35]. The fold change in normalized DYSF and MYOF values between case and control groups (the referent group being the control group in each instance) was calculated using the comparative C_T method and expressed as $2^{-\Delta\Delta C_T}$ [36]. In control reactions, it was confirmed that there was no amplification when the reverse transcriptase step was omitted.

Protein Extraction and Immunoblotting

Placental tissue (~60–120 mg) was pulverized under liquid N₂ using a mortar and pestle and subsequently incubated for 20 min in ice-cold octylglucoside lysis buffer (150 mM Na₂PO₄, 60 mM n-octyl β-D-glucopyranoside, 10 mM D-gluconic acid lactone, 1 mM EDTA) containing protease inhibitor cocktail. The lysates were clarified by centrifugation for 10 min at 14,000 × g and 4°C, and the supernatants were retained. Aliquots of each sample were assayed for protein concentration using the Bio-Rad protein dye reagent (Bio-Rad) using a standard of bovine serum albumin.

Equal amounts of total protein (40 µg/lane for DYSF and 80 µg/lane for MYOF) from each placental extract were resolved by SDS-PAGE using continuous 10% acrylamide gels (Thermo Scientific). Proteins resolved by gel electrophoresis were transferred electrophoretically to nitrocellulose membranes. Membranes were washed in Tris-buffered saline containing 0.2% Tween 20 (TBST), incubated for 1 h at 22°C with 5% nonfat milk in TBST, and then incubated overnight at 4°C with primary antibodies in TBST/milk. The antibody dilutions were anti-DYSF, 1:5000, and mouse polyclonal anti-MYOF, 1:1000. After washing in TBST, membranes were incubated with species-appropriate HRP-labeled secondary antibodies (diluted 1:2000) in TBST/milk for 1 h at 22°C. Antibody binding was detected using SuperSignal chemiluminescent substrate (Thermo Scientific) and detected using a VersaDoc Imaging System (Bio-Rad, Hercules, CA). Following detection of DYSF and MYOF, blots were subsequently reprobed using anti-GAPDH, 1:1000 as a control for protein loading. All chemiluminescent signals were collected within the linear response range of the CCD camera and analyzed using Quantity One software (Bio-Rad). The potential for cross-reactivity between the anti-DYSF and anti-MYOF antibodies was assessed in immunoblot assays [23, and unpublished observations], and was found to be negligible.

Immunofluorescence Microscopy (IFM)

Placental specimens were fixed for 1–2 h at room temperature in 4% paraformaldehyde (PFA)/PBS. To facilitate cryopreservation, the specimens were infiltrated with 20% sucrose/PBS overnight at 4°C. Specimens were cut into small pieces and embedded in Tissue-Tek Optimal Cutting Temperature compound (Electron Microscopy Sciences). Embedded tissues were frozen on dry ice and stored at –80°C until the time of sectioning (performed by the Pathology Core Facility, The Ohio State University, Columbus, OH). Cryostat sections (~6 µm) were stained with hematoxylin/eosin (to evaluate overall morphological integrity). For immunolabeling, unstained cryosections first were equilibrated in PBS at room temperature for 30 min and then incubated in 0.5% SDS in PBS for 10 min at room temperature for antigen retrieval. Sections washed free of SDS were next incubated in blocking solution (5% non-fat dry milk in PBS) for 1 h at room temperature before the addition of antibodies. The sections were then incubated with primary antibodies overnight at 4°C. The primary antibodies used included monoclonal anti-DYSF (1:100 dilution), mouse polyclonal anti-MYOF (1:50), and rabbit polyclonal anti-MYOF (1:50). The sections were subsequently washed and then incubated with fluorochrome-labeled secondary antibodies diluted 1:200 for 60 min at room temperature. After washing in PBS, the nuclei were stained with 5 µg/ml 4', 6-diamidino-2-phenylindole (DAPI) for 15 min before mounting in the ProLong anti-photobleaching agent. In control preparations, the primary antibodies were omitted, whereas the secondary antibody incubation was retained. As has been previously reported [22;23], these control preparations lacked appreciable immunolabeling, confirming that each of the primary antibodies provided specific immunolocalization patterns (data not shown). Cross-reactivity between the anti-DYSF and mouse polyclonal anti-MYOF antibodies by IFM was found to be insignificant in pilot studies using BeWo, JEG-3, and JAR cells [23]. In addition, double-labeling experiments using anti-DYSF and mouse polyclonal anti-MYOF antibodies revealed distinct labeling patterns in villous placenta sections (not shown), whereas parallel experiments using a combination of mouse and rabbit polyclonal anti-MYOF antibodies demonstrated identical labeling patterns.

Conventional fluorescence images were collected with a Zeiss Axiovert microscope equipped with a Photometrics Cool Snap HQ² CCD camera (Roper Industries, Pleasanton, CA). Images were captured using Slidebook image analysis software (Version 4.2, Intelligent Imaging Innovations, Denver, CO). All images were collected within the linear response range of the CCD camera. Exported micrographs were compiled with Photoshop CS2 software (Version 9, Adobe Systems).

Statistical Analyses

Statistical analyses were performed using Stata/IC version 10.0 (StataCorp, College Station, TX) and GraphPad Prism version 5.01 (GraphPad Software, La Jolla, CA) software packages. A Shapiro-Wilk test for normality was initially performed on DYSF and MYOF protein and mRNA expression data to confirm the validity of parametric statistical testing. The paired t-test was used to compare expression levels in sets of control and PE placentas matched for gestational age at delivery. To compare demographic and clinical characteristics between the groups, Student's t-test was used for parametric continuous variables, the Wilcoxon rank-sum test was used for non-parametric continuous variables, and Fisher's exact test was used for categorical variables (Table). Linear regression analysis was performed to test the association between DYSF or MYOF immunoreactivity and gestational age at delivery. In all instances, a p-value <0.05 was considered significant.

Results

Clinical Characteristics of Tissue Donors

Villous placental specimens were collected from 10 cases with severe preeclampsia (PE) and 10 controls matched for gestational age at delivery (± 7 days). All cases had proteinuric hypertension (an increase in blood pressure to at least 140 mm Hg systolic or 90 mm Hg after the 20th week of gestation, combined with urinary protein excretion of at least 0.3 g per 24 hours) in the presence of one or more of the diagnostic criteria for severe PE established by the National High Blood Pressure Education Program Working Group [34]. Specific diagnoses of severe PE were based upon: blood pressure criteria alone (2 cases); thrombocytopenia with laboratory evidence of hepatic dysfunction (1 case); central nervous system (CNS) symptomatology alone (1 case); and blood pressure criteria in addition to proteinuria exceeding 5 g per 24 h, CNS symptomatology, and/or laboratory evidence of hepatic dysfunction (6 cases). In one case, fetal growth restriction was suspected based on third-trimester ultrasound biometric parameters; however, at delivery, the infant was found to have a birthweight appropriate for gestational age. As such, no placentas from cases with intrauterine growth restriction (IUGR) were included in the present analysis.

Selected clinical and demographic data for the severe PE and control groups have been summarized (Table). The two groups were similar with respect to maternal age, gestational age at delivery, infant birthweight, and maternal race. Relative to the control group, subjects with severe PE were more likely to be nulliparous and primigravid. A similar percentage of controls and cases with severe PE were delivered in the absence of labor. A greater proportion of subjects with severe PE were delivered by cesarean section relative to the controls, although this difference was not statistically significant. As expected, the subjects with severe PE exhibited higher maximal systolic and diastolic blood pressures relative to the control group.

Expression of Placental DYSF and MYOF Proteins in Severe Preeclampsia

We first examined the relative expression of placental DYSF and MYOF proteins in each group by immunoblot analysis. Using the mouse monoclonal anti-DYSF antibody (Ham1/7B6), a single immunoreactive band with a molecular mass of ~ 230 kDa was observed (Fig. 1A). Similarly, when detected using a mouse polyclonal antibody, MYOF appeared as a single band also migrating at ~ 230 kDa (Fig. 2A). In the case of DYSF, pilot experiments demonstrated that delays in sample processing were accompanied by a decrease in the intensity of the major high molecular weight band, coupled with the appearance of multiple lower molecular weight species thought to represent degradation products (Supplemental Fig. 1). Importantly, no prominent lower molecular weight bands were observed in the current series of samples, indicating that sample integrity was sufficiently preserved using the collection methods described to enable reliable quantification.

By immunoblot analysis, placental DYSF expression was 38% lower on average among subjects with severe PE relative to control specimens (Fig. 1B; $p = 0.026$, paired t-test). Although a trend toward decreased MYOF levels in the PE placentas was observed, this was not statistically significant (Fig. 2B). In a prior report, quantitative IFM revealed that DYSF protein expression was approximately three times higher in first-trimester relative to term placentas [22]. We therefore performed linear regression to determine whether changes in expression of placental DYSF and MYOF were also present within the third trimester and could be correlated to gestational age at delivery. We observed no significant diminution in immunoreactive DYSF or MYOF expression with advancing gestational age in placental specimens delivered between 28 and 37 weeks of gestation (Supplemental Fig. 2). This result suggests that overall expression levels of DYSF and MYOF are relatively stable during the

third trimester. Further, the data presented in this regression analysis reflect the overall decrease in DYSF expression levels of the PE samples in comparison to the control specimens.

Expression of Placental DYSF and MYOF mRNA Species in Severe Preeclampsia

We considered that the decrease in placental DYSF protein expression observed in the setting of severe PE might result from reduced gene transcription or increased protein deportation (e.g., during syncytial shedding), among other possibilities. To examine whether changes in the expression of DYSF and MYOF mRNA species accompanied changes at the protein level, we performed quantitative real-time RT-PCR on the placental samples from cases of severe PE and controls (Fig. 3).

Placental DYSF mRNA levels were 57% lower on average among subjects with severe PE as compared to normotensive subjects (Fig. 3A; $p = 0.03$, paired t-test). There were no statistically significant differences in placental MYOF mRNA levels between subjects with severe PE and normotensive controls (Fig. 3B). These results suggest that DYSF gene expression in the placenta is relatively decreased in the setting of severe PE, and is not accompanied by compensatory MYOF upregulation. The diminution in placental DYSF mRNA is consistent with (and likely accounts for much of) the observed decrease in immunoreactive DYSF in severe PE.

Distribution of Placental DYSF and MYOF in Severe Preeclampsia

To characterize the expression of DYSF and MYOF in human placental cells in pregnancies complicated by severe PE, IFM was applied to conventional ($\sim 6 \mu\text{m}$) cryostat sections. In pilot experiments, we observed that immunolabeling was highly dependent upon the duration of fixation (Supplemental Fig. 3), such that labeling intensity decreased dramatically with increasing time in fixative. Due to variations in fixation time (between 1 and 2 h) in 50% of the specimens collected, planned quantitative IFM was abandoned.

As we previously documented in term placentas [22], DYSF was expressed in the STB and, to a variable extent, fetal capillary endothelial cells (Fig. 4A). Within the STB, DYSF labeling was largely restricted to the apical plasma membrane, with lesser staining along the basal surface. Interestingly, we observed no significant difference in the general patterns of DYSF localization between PE cases (Fig. 4B) and controls (Fig. 4A), although the overall staining intensity was somewhat lower among the PE samples. Thus, severe PE was not associated with a perturbation in the normal trafficking of DYSF to the apical STB.

MYOF labeling was also evident in the STB (Fig 4C, D) and, to a lesser extent, villous stromal cells and capillary endothelial cells. MYOF intensity was particularly strong among certain cells immediately interior to the STB (double arrows in Figs. 4C and 4D), the identity of which has not been established. Equivalent results were obtained using either the monoclonal MYOF antibody described previously [23], or a polyclonal MYOF antibody which recently became available from a commercial source (Sigma) (data not shown). As with DYSF, we observed no significant changes in MYOF localization in the setting of severe PE as compared to control specimens by standard epifluorescence.

Discussion

Increased syncytial shedding [37], together with augmented epithelial denudation and ultrastructural abnormalities (e.g., loss or distortion of microvilli at the STB apical surface [3;31;32]), suggest that STB plasma membrane stability may be compromised in the setting of PE. The current study was designed to address whether changes in the amount and/or distribution of ferlin proteins might be associated with this increase in membrane dynamics.

We found that placental DYSF expression was reduced in subjects with severe PE relative to gestational age matched controls. By contrast, the expression of MYOF was not reduced significantly, although a slight trend toward decreased protein levels among the PE placentas was observed. No appreciable changes in the distribution of these proteins in severe PE could be discerned. These data suggest a role for DYSF in the stability of the apical plasma membrane of the STB and may account, at least in part, for the increased shedding of microparticles in PE.

While it remains to be established the extent to which DYSF and MYOF contribute to STB apical plasma membrane repair, in light of the function of ferlin family proteins in general, this is not an unreasonable speculation [23;24]. There is compelling evidence that DYSF is required for vesicle fusion during Ca^{2+} -induced skeletal muscle membrane repair [18;19], and MYOF has recently been implicated in the repair of the plasma membrane in cultured endothelial cells [38]. Structural homologs and paralogs of these proteins similarly orchestrate the fusion of membranous vesicles to cell membranes [12–15;21], suggesting a mechanistic theme among ferlin proteins that is consistent with the “patch hypothesis” for membrane repair [7;8]. The assertion that DYSF and MYOF are important for membrane integrity is reinforced by the observation of coincident expression of these proteins at high levels in the STB (this work, and [22;23]); this is to be contrasted with observations in skeletal muscle [39;40], in which DYSF expression alone predominates in multinucleate myofibers, while MYOF is expressed in mononuclear myoblasts. As such, the expression of these Ca^{2+} -dependent membrane repair proteins may be so important in STB that functional redundancy has been selected for during the evolution of primate placentation.

It is interesting to note that, like DYSF, other gene products that are highly expressed in trophoblast are also decreased in the setting of PE; examples include HLA-G [41], placental isoform of ferritin [42], syncytin-1 [43;44], and the GCM1 (glial cells missing) transcription factor [45]. As was suggested by Colbern and colleagues [41], these decreases may be related to an overall reduction in the amount of trophoblastic tissue in placentas from patients with PE. Such findings could be related to increased STB-derived microparticle shedding [27] or impaired CTB fusion [46]. In addition, a functional relationship among GCM1, syncytin-1, and DYSF might contribute to the current findings. In the BeWo choriocarcinoma cell line, mononuclear cells express little DYSF protein until they are induced to form fused, syncytial-like structures through the addition of forskolin, an adenylate cyclase activator [23]. The mechanism of syncytial formation following forskolin treatment in BeWo appears to be mediated through the cAMP/protein kinase A signaling pathway, which enhances GCM1-mediated transcriptional activation [47]. GCM1, in turn, regulates syncytin-1 gene expression [48], which promotes cell-cell fusion. Although the regulatory elements that control DYSF gene expression during syncytialization are unknown, available evidence suggests that DYSF upregulation is subsequent to the activation of GCM1 and syncytin-1 gene expression. Assuming that this model faithfully recapitulates the molecular events of STB formation *in vivo*, it is reasonable that decreased placental DYSF expression would accompany decreases in syncytin-1 and GCM1 in the setting of PE.

The reduction in mean DYSF expression levels among PE placental specimens was not accompanied by a discernable change in MYOF expression. In part, this finding might reflect a limitation due to the small sample size. *Post hoc* analysis revealed that 10 pairs was sufficient to detect the observed effect on immunoreactive DYSF expression with 82% power, while the power to detect the more modest reduction in immunoreactive MYOF was only 27%. The lack of a significant decrease in MYOF by immunoblot or real-time RT-PCR analysis might also reflect inherent limitations of these assays, which would tend to underestimate changes in MYOF expression if these were restricted to the STB. Of note, DYSF expression was relatively restricted, being confined largely to the apical STB and, to a lesser extent, fetal capillary

endothelial cells; by comparison, MYOF was expressed more broadly among placental cell types. As such, the DYSF expression levels obtained using these methods would be more representative of expression in STB and fetal capillary endothelial cells, whereas MYOF expression would reflect villous expression more broadly. As a consequence, particularly in light of the small sample size, it cannot be discerned from these methods alone whether MYOF expression was actually decreased, or possibly increased, in the STB specifically (as opposed to the placental villi overall). In future studies, a method such as quantitative IFM may provide a more reliable means to assess STB-specific MYOF expression in tissue specimens. However, any such studies must ensure that the specimen collection and fixation procedures are held strictly constant, since stability and immunoreactivity of the ferlin proteins in placental tissue are susceptible to proteolytic degradation and are sensitive to fixation conditions. Further, due to these limitations, careful interpretation of results obtained from retrospective studies of banked fixed tissue must also be considered.

The pathological significance of the observed association between decreased placental DYSF expression in PE requires further investigation. To date, the extent to which pregnancy complications affect mothers carrying fetuses with dysferlin deficiencies (such as those associated with limb girdle limb girdle muscular dystrophy type 2B and Miyoshi Myopathy) has not been addressed. At a minimum, the current data suggest that MYOF may be present in sufficient amounts to compensate for relative DYSF deficiency in the setting of PE. In addition, ferlin-independent compensatory membrane repair pathways might also be invoked in this context. As an example, in clinical cases of dysferlin deficiencies, it has been suggested that skeletal muscle (and possibly other tissues) may utilize a “rescue” membrane repair pathway involving the synaptotagmin like protein Slp2a, which has structural similarities to DYSF [49]. It is entirely plausible that a similar mechanism may be utilized by preeclamptic placentas. Alternatively, villous lesions may also be contained via the deposition of fibrin-rich fibrinoid at sites of STB denudation [50–52]. The utilization of such compensatory repair pathways, and the extent to which these may ameliorate (or exacerbate) pathological sequelae, remains to be determined.

In summary, the current work presents novel evidence suggesting that the expression of placental DYSF, but not placental MYOF, is decreased in the setting of severe PE. While this manuscript was in preparation, we became aware of the preliminary results of a second study in which the expression of DYSF and MYOF in preterm placentas from pregnancies affected by preeclampsia and intrauterine growth restriction was examined [53]. In agreement with our current results, this abstract suggested that DYSF, but not MYOF, mRNA levels were down-regulated in placentas in the setting of PE. To the best of our knowledge, these two reports represent the first to examine this relationship. Although the functional significance of these findings have yet to be corroborated, the current results suggest that relative DYSF deficiency is associated with (and possibly contributes to) STB plasma membrane instability in placentas complicated by PE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Expression of immunoreactive DYSF in subjects with severe preeclampsia ($n = 10$) and normotensive controls ($n = 10$) matched for gestational age at delivery. A) Representative immunoblots prepared from villous placental samples demonstrating the relative levels of expression of DYSF and GAPDH in control specimens and those delivered of subjects with severe PE. Four of the ten case-control pairs used in the analysis are shown. B) Values obtained from quantitative analysis of immunoreactive DYSF in placentas delivered of control (white bars) and preeclamptic (black bars) subjects. Data were normalized to GAPDH levels for each specimen and multiplied by a factor of ten to facilitate interpretation (mean $\pm$ SEM). Placental DYSF protein expression was significantly different between the groups, $P < 0.05$ (paired t-test).

Figure 2.

Expression of immunoreactive MYOF in subjects with severe preeclampsia ($n = 10$) and normotensive controls ($n = 10$) matched for gestational age at delivery. A) Representative immunoblots prepared from villous placental samples demonstrating the relative levels of expression of MYOF and GAPDH in control specimens and those delivered of subjects with severe PE. Four of the ten case-control pairs used in the analysis are shown. It should be noted that the apparent association between severe PE and reduced immunoreactive MYOF suggested in these immunoblots was not observed in all case-control pairs. B) Values obtained from quantitative analysis of immunoreactive MYOF in placentas delivered of control (white bars) and preeclamptic (black bars) subjects. Data were normalized to GAPDH levels for each specimen and multiplied by a factor of ten to facilitate interpretation (mean $\pm$ SEM). N.S., not significant (paired t-test).

Figure 3.

Expression of placental DYSF and MYOF mRNA species in subjects with severe preeclampsia ($n = 10$) and normotensive controls ($n = 10$) matched for gestational age at delivery. A,B) Real-time RT-PCR was used to assess DYSF (A) and MYOF (B) mRNA expression in placentas delivered of control (white bars) and preeclamptic (black bars) subjects. For each target gene, the threshold cycle (C_T) at which each mRNA species was detected was normalized to that of RPLPO. The fold-change in transcript expression was calculated in relation to the severe PE group (for DYSF) or control group (for MYOF) using the comparative C_T method [36], and expressed as mean $\pm$ SEM in the graphs. Only placental DYSF mRNA expression was significantly different between the groups, $P < 0.05$ (paired t-test). N.S., not significant.

Figure 4.

The localization of DYSF and MYOF within villous structures does not change in the setting of severe PE. A and B) Representative IFM localization of DYSF (red) in 6 μm sections of villous placenta from a normotensive subject (A) and a subject with severe PE (B) as observed using conventional epifluorescence microscopy. The most intense signal was found in the STB (the nuclei of which are denoted by single arrows), particularly in the apical plasma membrane regions (arrowheads). Labeling intensity was typically less prominent, but more variable, in fetal capillary endothelial cells (asterisks indicate lumens of vessels). There were no appreciable changes in the distribution of DYSF labeling in either setting. C and D) Representative IFM localization of MYOF (green) in 6 μm placental sections from a normotensive subject (C) and a subject with severe PE (D) as observed using conventional epifluorescence microscopy. MYOF labeling was prominent in STB (arrowheads), with less intense labeling in villous stromal cells and variable labeling in fetal capillary endothelial cells (asterisks). In most instances, cells interior to the STB exhibited pronounced MYOF labeling (double arrows). Nuclei counterstained with DAPI appear blue in all panels. Bars = 20 μm .

Table 1

Selected clinical characteristics of severe preeclamptic subjects and controls

	Control n = 10	Severe PE n = 10
Maternal age (y) *	26.2 ± 6.1	25.1 ± 5.6
Gravidity *	3.2 ± 2.3 **	1.4 ± 0.7 **
Parity *	1.3 ± 1.7 **	0.0 ± 0.0 **
Gestational age at delivery (weeks) *	34.0 ± 2.4	33.7 ± 2.4
Birthweight (g) *	2306.8 ± 504.9	2228.0 ± 669.7
Max. SBP (mm Hg) *	135.2 ± 18.9 †	177.1 ± 17.9 †
Max. DBP (mm Hg) *	83.3 ± 9.7 †	107.3 ± 9.5 †
No labor	20%	30%
Cesarean section	40%	70%
Maternal race		
White	70%	80%
Black	30%	20%

* Mean ± standard deviation

** denotes $p \leq 0.05$ † denotes $p \leq 0.01$

Abbreviations: SBP (systolic blood pressure), DBP (diastolic blood pressure)