Changes in Vasodilator-Stimulated Phosphoprotein Phosphorylation, Profilin-1, and Cofilin-1 in Accreta and Protection by DHA

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Mehboob Ali, PhD¹ , Lynette K. Rogers, PhD^{1,2},
Kathryn M. Heyob, MS¹, Catalin S. Buhimschi, MD, MBA³,
and Irina A. Buhimschi, MD^{1,2,3}

Abstract

Accreta and gestational trophoblastic disease (ie, choriocarcinoma) are placental pathologies characterized by hyperproliferative and invasive trophoblasts. Cellular proliferation, migration, and invasion are heavily controlled by actin-binding protein (ABP)-mediated actin dynamics. The ABP vasodilator-stimulated phosphoprotein (VASP) carries key regulatory role. Profilin-1, cofilin-1, and VASP phosphorylated at Ser157 (pVASP-S157) and Ser239 (pVASP-S239) are ABPs that regulate actin polymerization and stabilization and facilitate cell metastases. Docosahexaenoic acid (DHA) inhibits cancer cell migration and proliferation. We hypothesized that analogous to malignant cells, ABPs regulate these processes in extravillous trophoblasts (EVTs), which exhibit aberrant expression in placenta accreta. Placental–myometrial junction biopsies of histologically confirmed placenta accreta had significantly increased immunostaining levels of cofilin-1, VASP, pVASP-S239, and F-actin. Treatment of choriocarcinoma-derived trophoblast (BeWo) cells with DHA (30 μ M) for 24 hours significantly suppressed proliferation, migration, and pVASP-S239 levels and altered protein profiles consistent with increased apoptosis. We concluded that in accreta changes in the ABP expression profile were a response to restore homeostasis by counteracting the hyperproliferative and invasive phenotype of the EVT. The observed association between VASP phosphorylation, apoptosis, and trophoblast proliferation and migration suggest that DHA may offer a therapeutic solution for conditions where EVT is hyperinvasive.

Keywords

placenta accreta, extravillous trophoblast, actin-binding proteins, DHA

Introduction

Human placentation involves establishment of an efficient attaching system and blood flow between mother and its fetus. The anchoring villi, which are tunnel-like structures, hold the placenta in close contact to the myometrium. During this tightly regulated process, trophoblasts migrate from the basement membrane, adhere to and penetrate the decidua, and ultimately invade the inner third of the myometrium and the arterioles supplying this region.^{1–3} Placental vasodilator-stimulated phosphoprotein (VASP) is expressed throughout pregnancy, with notably high expression in invasive extravillous trophoblasts (EVTs) of anchoring villi and distal columns.⁴ Aberrant EVT proliferation, migration, and invasion into uterine tissue have been observed in placenta disorders characterized by both insufficient (preeclampsia) and excessive (accreta) invasion.^{5–11} In cancer cell proliferation and migration, which closely resemble placenta trophoblast hyperproliferative diseases, differential phosphorylation of VASP at

Ser157 (pVASP-S157) and Ser239 (pVASP-S239), profilin-1, and cofilin-1 mediate cellular actin cytoskeletal remodeling of proliferating and migrating cancer cells.^{12–19} These proteins and their possible interactions during actin remodeling further regulate the formation of protrusive structures such as lamellipodia and filopodia (horizontally) and invadopodia (vertically down the nuclear axis),^{16,17,19–23} which facilitate cell migration

¹ Center for Perinatal Research, The Research Institute at Nationwide Children's Hospital, Columbus, OH, USA

² Department of Pediatrics, The Ohio State University College of Medicine, Columbus, OH, USA

³ Division of Maternal-Fetal Medicine, Department of Obstetrics & Gynecology, The Ohio State University College of Medicine, Columbus, OH, USA

Corresponding Author:

Mehboob Ali, Center for Perinatal Research, The Research Institute at Nationwide Children's Hospital, 575 Children's Crossroad, Columbus, OH 43215, USA.

Email: mehboob.ali@nationwidechildrens.org

and invasion.^{20,24-27} To date, these mechanisms have not been explored with respect to EVT hyperproliferation and migration associated with placenta accreta.

Actin remodeling (polymerization, stabilization/depolymerization) is regulated by VASP, profilin-1, and cofilin-1 in a feedback mechanistic manner. Vasodilator-stimulated phosphoprotein is an actin-binding protein (ABP) that contains 2 Ena/VASP homology (EVH) domains separated by a central proline-rich region (PRR). Cyclic adenosine monophosphate-dependent protein kinase preferentially phosphorylates Ser157 within the PRR, increasing actin binding activity and promoting formation of cytoskeleton-shaped membrane protrusions.²⁸ In contrast, protein kinase-G preferentially phosphorylates Ser239 within the C-terminal EVH2 domain, which promotes F-actin dissociation and inhibits cytoskeleton-shaped membrane protrusions.^{16,19,29,30} Profilin-1 is a ubiquitous ABP that regulates actin polymerization in response to extracellular signals.^{18,24,31} Cofilin-1 (nonmuscle; n-cofilin) is an actin-modulating member of the actin depolymerizing factor/cofilin family widely distributed in intracellular regions that binds to and depolymerizes filamentous F-actin and inhibits polymerization of monomeric G-actin.^{26,32,33} Cofilin-1 has roles in actin dynamics depending upon concentration, phosphorylation status, G-actin, other protein factors, and cellular functional requirements.³⁴⁻³⁹ Moreover, cofilin-1 levels vary among cancer cell and tumor types and degree of proliferation and migration.^{26,40-42}

Docosahexaenoic acid (DHA), an anti-inflammatory omega-3 fatty acid often taken as a dietary supplement, has been suggested as a pharmacologic agent in placental disorders, which are often associated with inflammation.⁴³⁻⁴⁷ This suggestion has pathophysiologic plausibility because DHA inhibits cancer cell proliferation, migration, invasion, and survival.^{12,48,49} We recently showed that the antiproliferative, antimigratory, and anti-invasiveness effects of DHA in cancer cells were mediated through altered profilin-1 and cofilin-1 expression and differential phosphorylation of VASP.¹² Here, we tested whether DHA modulates trophoblast hyperproliferation and migration through actin homeostasis. Differential VASP phosphorylation and ABP expression in human placenta biopsy tissue were associated with placenta accreta. In vitro experiments explored these relationships with respect to trophoblast proliferation and migration in the context of DHA treatment.

Materials and Methods

Patients and Biological Samples

We analyzed placental-myometrial biopsies retrieved from 12 women who had a hysterectomy due to suspected aberrant invasive placentation enrolled at Yale-New Haven Hospital between May 2005 and July 2012. Our study groups included 8 women who were found to have clinically adherent placenta upon delivery (gestational age [GA], median [range]: 30 [20-36] weeks).

The decision for hysterectomy was taken independent of our research protocol. Following hysterectomy, a full-thickness myometrial-placental biopsy was retrieved in sterile conditions. Examination of the hysterectomy surgical specimens by a perinatal pathologist confirmed 4 placenta accreta (2 increta and 2 percreta) with villi directly implanted into the myometrium without an intervening layer of decidua. Four cases had focal accreta requiring hysterectomy. For comparison, placental bed biopsies and placental villous tissue of women (n = 4, GA: 37 [36-39] weeks) with an uncomplicated pregnancy and elective term cesarean delivery were used as control.

All women who provided specimens signed informed consent under research protocols approved by Human Investigation Committees at Yale University. The Human Investigation Committees at The Ohio State University and The Research Institute at Nationwide Children's Hospital approved our research.

Immunofluorescence Staining of Placenta Accreta Biopsy Samples to Assess ABP Expression

Tissues of the uteroplacental junction were fixed in formalin, embedded in paraffin, and cut as serial sections at 5 μ m thickness. Region of interest on each slide was identified by clusters of EVTs that displayed characteristics reported by us in prior studies such as coexpression of vimentin and cytokeratin and loss of E-cadherin.^{50,51} Slides were processed using standardized protocols for immunofluorescence labeling with antibodies targeting profilin-1, cofilin-1, VASP, and pVASP-S239 (1:500; Cell Signaling Technology, Inc, Danvers, Massachusetts) and pVASP-S157 (1:500; Santa Cruz, Dallas, Texas). Slides were then stained with anti-rabbit IgG Alexa Fluor 488 conjugate (1:1000; Cell Signaling Technology) and nuclei counterstained with DAPI (Invitrogen, Carlsbad, California). A minimum of 4 representative images from each slide were taken using a Zeiss Axio Scope.A1 Polarizing Microscope (Carl Zeiss, Jena, Germany) with 400 $\times$ magnification using identical settings. Image color intensity was quantified using NIH ImageJ 1.47v software using identical background settings.

F-Actin Content

Tissular F-actin content was estimated using Alexa Fluor 633 phalloidin (Invitrogen, Eugene, Oregon) and nuclei counterstained with DAPI. Images were acquired as described above, maintaining identical settings between samples, and F-actin content quantified using NIH ImageJ.

Cell Culture and Treatment

Trophoblast-derived choriocarcinoma cells (BeWo; American Type Culture Collection, Manassas, Virginia) were cultured (at 37°C) in a humidified atmosphere containing 5% CO₂ using DMEM/F-12 (1:1) 1 $\times$ with 10% FBS and supplemented with 2.438 g/L sodium bicarbonate (Gibco, Thermo Scientific, Gaithersburg, Maryland). Cells (>95% viable; checked by

trypan blue exclusion) at passage #4 were treated at ~80% confluence for 24 hours with DHA (30 μ M) or phosphate-buffered saline (PBS; vehicle control). Docosahexaenoic acid (30 μ M) was used to examine effects on key trophoblast apoptotic proteins, a concentration previously found to significantly change expression levels of key apoptosis marker proteins in MCF7 breast cancer cells.⁴⁸

Proliferation (Cell Growth Assay)

Cells were treated with DHA or PBS for 24 hours, dissociated with 0.25% trypsin EDTA (Mediatech, Inc, Corning Life Sciences, Manassas, Virginia), and counted using a Neubauer chamber. Equal numbers of cells were plated in 6-well plates. Proliferation (cell growth) was assessed by counting cells at 2, 4, and 6 days after dissociation and is expressed as total number of cells at each time point.

Migration (Wound Assay)

To assess cell migration, BeWo cells were plated in 6-well plates, grown until ~60% confluent, and then treated for 24 hours with DHA or PBS prior to wound induction. Five wounds per treatment were randomly marked with permanent marker and photographed at 0, 24, and 48 hours with an Olympus BX51 compound microscope (Olympus, Center Valley, PA) at 10 $\times$ magnification. NIH ImageJ software was used to measure the distance between the wound edges as described.¹²

Quantification of BeWo Cell ABP and Apoptosis Protein Expression

For analysis of ABPs, total protein from BeWo cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies targeting profilin-1, cofilin-1, VASP, pVASP-S157, and pVASP-S239 (dilution, 1:1000) and anti- β -actin mouse monoclonal antibodies (1:10 000; Abcam, Cambridge, Massachusetts). Cell lysates were similarly analyzed for expression of apoptosis proteins caspase-9, BCL-2, and Bax using rabbit polyclonal anticaspase-9 and anti-BAX antibodies and mouse monoclonal anti-BCL-2 antibody (1:500; Cell Signaling Technology). Membranes were then probed with horseradish peroxidase-conjugated antirabbit or antimouse secondary antibody (dilution, 1:1000; BD Pharminogen, Franklin Lakes, New Jersey) for 1 hour at room temperature. Bands were visualized using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, United Kingdom); band intensity measured by densitometry and normalized to β -actin; pVASP was additionally normalized to total VASP.

Statistical Analysis

Data were analyzed by *t* test, 1-way analysis of variance (ANOVA) or 2-way repeated-measures ANOVA and further

analyzed by Tukey post hoc test. Cell culture experiments were repeated 3 times using triplicate in each time and data are presented as mean $\pm$ standard error of the mean. Data analysis was performed with Prism version 7.03 (GraphPad Software, La Jolla, California). A value of $P < .05$ was considered statistically significant.

Results

Placenta Accreta Tissue Had Increased F-Actin and Altered ABP Expression

Figure 1A shows representative images of normal and pathological placenta biopsy tissues stained for F-actin and ABPs. Average protein expression intensity is depicted adjacent to images of placental sections stained with the relevant antibody (Figure 1B-H). Placenta accreta had significantly increased F-actin compared with normal placentation (Figure 1B, $P = .018$). Compared with healthy control placenta, accreta had significantly increased levels of VASP (Figure 1C, $P = .030$), cofilin-1 (Figure 1D, $P = .019$), and pVASP-S239 (Figure 1E, $P = .022$). In contrast, focal accreta only had significantly increased levels of cofilin-1 compared to normal placentation (Figure 1D, $P < .001$). No differences in profilin-1 (Figure 1F) or pVASP-S157 (Figure 1G) levels were observed in accreta or focal accreta compared to healthy placenta.

Docosahexaenoic Acid Reduced Proliferation and Migration of BeWo Cells

Using BeWo cells as a model trophoblast system, DHA treatment for 24 hours suppressed BeWo cell proliferation to a level that reached significance by 6 days posttreatment (Figure 2A, $P < .001$). Using a wound assay, wound closure was nearly complete in the control by 48 hours (Figure 2B). However, there was significantly reduced trophoblast migration at 24 hours ($P < .05$) and 48 hours ($P < .001$) following treatment with DHA (Figure 2C). There was a significant interaction between treatment and time for both proliferation ($P < .001$) and migration ($P = .038$).

Docosahexaenoic Acid Alters the Expression Profile of ABPs in BeWo Cells

To determine whether the DHA effects on trophoblast proliferation and migration were associated with changes in ABP expression patterns, total protein isolated from BeWo cells treated with DHA or PBS for 24 hours was analyzed by Western blot. At the tested concentration, DHA had no significant effects on VASP (Figure 3A), profilin-1 (Figure 3B), or cofilin-1 (Figure 3C) expression levels. Docosahexaenoic acid differentially affected VASP phosphorylation, with significantly reduced phosphorylation at Ser239 (Figure 3D, $P = .022$), but no effect on phosphorylation at Ser157 (Figure 3E).

Figure 1. Placenta accreta expresses high levels of F-actin, cofilin-I, VASP, and pVASP-S239. F-actin content, profilin-I and cofilin-I expression, and VASP phosphorylation were quantified in term human placenta biopsy samples from normal healthy controls, focal accreta, and accreta using florescent microscopy (A, 400 $\times$ magnification) and NIH ImageJ analysis (B-G). A, F-actin (red) stained with phalloidin; profilin-I, cofilin-I, VASP, pVASP-S239, and pVASP-S157 (green) stained with specific antibodies; nuclei (blue) counterstained with DAPI. Data were analyzed by *t* test followed by 1-way ANOVA and further analyzed by Tukey post hoc. (*) $P < .05$, and (***) $P < .001$, in comparison to control; $n = 4$ tissue sections from different donors. Scale bar is 200 μ m. ANOVA indicates analysis of variance; VASP, vasodilator-stimulated phosphoprotein.

Docosahexaenoic Acid Induces Apoptosis in BeWo Cells

Docosahexaenoic acid significantly induced expression of the proapoptotic caspase-9 protein in BeWo cells 24 hours after treatment (Figure 4A, $P = .005$), concurrently suppressed

expression of the antiapoptotic BCL-2 protein (Figure 4B, $P = .009$), and had no effect on proapoptotic Bax protein expression (Figure 4C). Collectively these results indicate that DHA can induce trophoblast apoptosis.

Figure 2. Docosahexaenoic acid supplementation inhibits proliferation and migration of trophoblast-derived choriocarcinoma cells, BeWo. A, BeWo cell proliferation based on cell counts taken at 2, 4, and 6 days following 24 hours treatment with 30 μ M DHA or PBS (Control). B, Representative wound assays of BeWo cells treated for 24 hours with 30 μ M DHA or PBS prior to wound induction using a 20 μ L pipette tip. C, Cell migration (percent wound closure) was quantified from digital images of the same regions taken at 0, 24, and 48 hours posttreatment. Images were captured with an Olympus BX51 compound microscope at 10 $\times$ magnification and analyzed with NIH ImageJ software. Data were analyzed by 2-way repeated measures ANOVA and by Tukey post hoc. (*) $P < .05$, and (***) $P < .001$, $n = 3$ experiments. Scale bar is 200 μ m. ANOVA indicates analysis of variance; DHA, docosahexaenoic acid; PBS, phosphate-buffered saline.

Discussion

Extravillous trophoblast migration and invasion are highly regulated and critical for successful placentation. Unsurprisingly, there is an incomplete understanding of the numerous factors that contribute to its deregulation, resulting in pathological conditions^{5,10,52} of hyper- (placenta accreta or choriocarcinoma) and hypoinvasion (preeclampsia and intrauterine growth restriction).^{5,6,10,43} Interpretation of the existing data suggests that reestablishing homeostasis earlier in pregnancy during the hyperproliferative trophoblast stage might offer a novel approach to target overinvasive disorders (eg, accreta, percreta, and increta).¹¹ However, before beginning to test clinically DHA's potential therapeutic role, we should first identify key proteins and molecules participating in regulation of EVT proliferation, migration, and invasion.

Actin dynamics regulated through ABPs mediate cancer cell proliferation, migration, and invasion during metastasis^{17,20,27} and may be similarly associated with these processes in trophoblasts. Previous data demonstrating temporal changes in placental trophoblast VASP expression and phosphorylation during the course of normal pregnancy suggest an intrinsic role for this phosphoprotein regulating trophoblast function.⁴ However, phosphorylation specificity was not yet determined. As in cancer cells, dysregulated trophoblast VASP expression or activation (phosphorylation) might be associated with aberrant proliferation and migration, facilitating placental hyperinvasion during accreta. Our assertion is consistent with prior studies demonstrating differential VASP phosphorylation and altered profilin-1- and cofilin-1-mediated actin dynamics regulate proliferation and migration.^{13,15,39,40,53} Our results demonstrating increased VASP, pVASP-S239, and cofilin-1 in the context of increased F-actin are suggestive of altered

Figure 3. Docosahexaenoic acid modulates expression of actin-binding proteins in trophoblast-derived choriocarcinoma cells. BeWo cells pretreated for 24 hours with 30 μ M DHA or PBS (vehicle control) were harvested and total protein subjected to Western blot analysis of (A) VASP, (B) profilin-1, (C) cofilin-1, (D) pVASP-S239, and (E) pVASP-S157. Data were analyzed by *t* test followed by 1-way ANOVA and by Tukey post hoc. (*) $P < .05$, (**) $P < .005$, and (***) $P < .001$, $n = 3$ experiments. ANOVA indicates analysis of variance; DHA, docosahexaenoic acid; PBS, phosphate-buffered saline; VASP, vasodilator-stimulated phosphoprotein.

Figure 4. Docosahexaenoic acid induces apoptosis in trophoblast-derived choriocarcinoma cells. BeWo cells were treated with DHA for 24 hours and harvested for Western blot analysis of apoptosis proteins (A) caspase-9, (B) BCL-2, and (C) Bax. Data were analyzed by *t* test followed by 1-way ANOVA and further analyzed by Tukey post hoc. (*) $P < .05$, (**) $P < .005$, $n = 3$ experiments. ANOVA indicates analysis of variance; DHA, docosahexaenoic acid.

actin dynamics as a mechanism regulating trophoblast hyperproliferation and migration through promotion of homeostasis.

Fundamental to understanding accreta pathology is determining whether dysregulated ABPs are causal or a response to trophoblast hyperproliferation and migration. Cofilin-1 participates in actin polymerization by providing adenosine triphosphate-actin monomers to profilin-1 for production of

F-actin^{34,37}; elevated levels suggest potential cofilin-1 involvement in increased F-actin content in accreta and in trophoblast hyperproliferation and migration. Cofilin-1 is associated with certain cancers^{26,54-56} and its overexpression and silencing, respectively, exacerbate or abrogate cancer cell hyperproliferation, migration, and invasion, although absence of parallel investigation of actin remodeling and profilin-1 renders

comparisons to accreta and trophoblasts incomplete.^{57,58} Previous studies showed increased F-actin, high proliferation, and migration require active participation of profilin-1 and pVASP-S157 in actin dynamics,^{17,19,24,40,53} although neither of these proteins were changed in our study, despite high levels of F-actin at the late stage of disease. One interpretation of our results is that unchanged levels of profilin-1 and pVASP-S157 in accreta biopsy tissue represent a return of proactin polymerization machinery to basal levels at this stage of disease, with elevated cofilin-1 favoring actin depolymerization mechanics stabilizing actin.^{39,59} However, we cannot exclude the possibility that lack of change in profilin-1 and pVASP-S157 in accreta and unresponsiveness to DHA in vitro might reflect differences in regulation of overinvasiveness between trophoblasts and cancer cells.

Treatment of 3 prostate cancer cell lines with 23,24-dihydrocucurbitacin F suggests antiproliferative effects can occur through actin aggregation and cofilin-actin rod formation, leading to apoptosis.⁶⁰ Significantly increased pVASP-S239 levels in cancer cells and tissue counter high actin polymerization, hyperproliferation, invasion, and migration.^{13,15,40,61} As shown by our data, increased levels of VASP, pVASP-S239, and cofilin-1 in the context of increased F-actin content suggest a regulatory role of these proteins to counteract trophoblast hypermigration and proliferation.

Docosahexaenoic acid reduces cancer cell aggressiveness, which is associated with changes in actin dynamics and apoptotic profiles.^{40,48} Similar effects on the proliferative and migratory activity of trophoblasts (BeWo) seen through our experiments suggest involvement of common target(s) and machinery. Contrasting with cancer cells,^{40,61} reduced proliferation and migration in BeWo cells treated with DHA were associated with a parallel decrease in VASP S239 phosphorylation and cofilin-1 level that trended lower. Although much awaits discovery, as in cancer studies, DHA has shown both its potential to temper trophoblast invasive phenotype and its utility as a tool to manipulate ABP expression patterns. Opposite effects on BeWo ABP expression than observed in cancer cells might be related to DHA levels, although we cannot exclude cell-specific differences in responsiveness to DHA.

Evidence from cancer studies associates cofilin-1 with increased proliferation and reduced apoptosis.⁵⁶⁻⁵⁸ Significantly induced expression of proapoptotic caspase-9 and suppression of antiapoptotic BCL-2, with cofilin-1 trending lower, suggest DHA-modulating effects similar to cofilin-1 knock-down in bladder cancer cells⁵⁶ and suggests that reduced BeWo cell growth was at least partially due to increased apoptosis. Because accreta tissue are characterized by increased trophoblast proliferation regardless of severity,¹¹ inducing apoptosis in overproliferative trophoblasts may have therapeutic benefit.

Conclusion

Our study provides evidence that ABPs are active participants in the molecular events regulating the invasive phenotype of the EVT. We propose that increased VASP Ser239

phosphorylation and cofilin-1 might counteract elevated F-actin and high trophoblast proliferation in accreta. In vitro data support proposed roles for DHA-mediated pVASP-S239/cofilin-1 expression and apoptosis, which affected BeWo cell migration and proliferation, respectively; although more in-depth investigation is needed to explore a connection between therapeutic potential of DHA and ABPs in overinvasive disorders.

Authors' Note

M.A. conceived the ideas, designed, executed the experiments, wrote the manuscript, and approved the final draft. K.M.H. helped in in vitro culture studies. L.K.R. assisted with writing the manuscript. I.A.B and C.S.B helped in providing the translational character of this study by providing the human biopsy tissues, analyzing the data in a critical fashion, and editing of the manuscript.

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Declaration of Conflicting Interests

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ORCID iD

Mehboob Ali, PhD <https://orcid.org/0000-0003-1657-7245>

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