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The invasive phenotype of placenta accreta extravillous trophoblasts associates with loss of E-cadherin

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ABSTRACT

Introduction: Epithelial-to-mesenchymal transition (EMT) is a process of molecular and phenotypic epithelial cell alteration promoting invasiveness. Loss of E-cadherin (E-CAD), a transmembrane protein involved in cell adhesion, is a marker of EMT. Proteolysis into N- and C-terminus fragments by ADAM10 and presenilin-1 (PSEN-1) generates soluble (sE-CAD) and transcriptionally active forms. We studied the protein expression patterns of E-CAD in the serum and placenta of women with histologically-confirmed over-invasive placentation.

Methods: The patterns of expression and levels of sE-CAD were analyzed by Western blot, immunoassay, and immunoprecipitation. Tissue immunostaining for E-CAD, cytokeratin-7 (epithelial marker), vimentin (mesenchymal marker), ADAM10, PSEN-1 and β-catenin expression were investigated in parallel.

Results: N-terminus cleaved 80 kDa sE-CAD fragments were present in serum of pregnant women with gestational age regulation of the circulatory levels. Women with advanced trophoblast invasion did not display circulatory levels of sE-CAD different from those of women with normal placentation. Histologically, extravillous trophoblasts (EVT) closer to the placental–myometrial interface demonstrated less E-CAD staining than those found deeper in the myometrium. These cells expressed both vimentin and cytokeratin, an additional feature of EMT. EVT of placentas with advanced invasion displayed intracellular E-CAD C-terminus immunoreactivity predominating over that of the extracellular N-terminus, a pattern consistent with preferential PSEN-1 processing.

Discussion: Local processing of E-CAD may be an important molecular mechanism controlling the invasive phenotype of accreta EVT.

1. Introduction

The incidence of excessively invasive placentation (placenta accreta) has increased in recent years to a rate of ~1:533 deliveries [1]. Although accreta is a major cause of maternal morbidity and mortality, few studies have explored the molecular mechanisms responsible for placental over-invasion [2–4]. Hypotheses focusing on myometrial scar [5], absent protective decidua [5], or aberrant angiogenesis at the implantation site [6], have only partially explained the aggressive behavior of the invasive trophoblast.

Epithelial-to-Mesenchymal Transition (EMT) is a highly conserved developmental program that converts immotile epithelial cells into migratory mesenchymal cells [7]. When activated, EMT causes modified cell polarity, decreased adhesion, and increased mobility [8]. EMT is an integral part of implantation and

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early placental development [8], allowing assembling of trophoblast cell columns that invade decidual stroma in the late first and early second trimester [9,10]. Once the process of implantation is complete, the cellular EMT process ceases and the extravillous trophoblast (EVT) revert back to an epithelial phenotype [10]. We previously reported that accreta EVT display features consistent with EMT based on co-expression of vimentin with cytokeratin [11]. Because this association is a hallmark of EMT [12], and of a metastable phenotype [13], we proposed EMT is part of the regulatory mechanisms impacting the migratory behavior of the trophoblast of women with placenta accreta.

E-cadherin (E-CAD) is a Ca^{2+} dependent single-pass transmembrane cell–cell adhesion molecule that functions as caretaker of an epithelial phenotype [14]. Loss of E-CAD is a key EMT event leading to aberrant cell adhesion and initiation of an invasive phenotype [15]. Correspondingly, the migratory phenotype of trophoblast has been associated with down-regulation of E-CAD during *in-vitro* co-culture of first trimester placental villi and decidua parietalis [16]. Down-regulation in E-CAD expression involves generation of extracellular N-terminus and intracellular C-terminus cleavage fragments via proteolysis by ADAM10 [17] and presenilin-1 (PSEN-1)/γ-secretase system, respectively [18]. C-terminus bound β-catenin, which has connective roles between E-CAD and the actin cytoskeleton, is subsequently dislocated, impacting cell motility.

In this study we aimed to explore the process of EMT in placenta accreta, by investigating maternal circulatory levels of sE-CAD. We further evaluated the tissue expression of E-CAD and its associated proteins in women with over-invasive placentation.

2. Methods

2.1. Patient population and serum samples

We analyzed 146 serum samples retrieved from 84 women enrolled at Yale-New-Haven-Hospital between May 2005 and July 2012. Our study group included serum and histological samples from 23 women who had suspected over-invasive placentation based on antepartum sonography [19] and who were found upon delivery to have a morbidly adherent placenta [gestational age (GA), median, interquartile range [IQR]: 29 [24–35] weeks]. Of these women, 5 had placenta accreta, 11 increta and 7 percreta. Details about clinical groups, and samples retrieved as controls are presented in the Supplementary Fig. 1. All women signed informed consent under protocols approved by the Human Investigation Committee of Yale University.

2.2. Blood collection, storage

Blood collection was performed at the time of admission for vaginal bleeding or prospectively during an office visit. Samples were retrieved and processed as previously described [11].

2.3. sE-CAD immunoassay, Western blotting and immunoprecipitation

ELISA assays for human serum sE-CAD (N-terminus, R&D Systems, Minneapolis, MN) were performed following 1:20 sample dilution. Minimum detection level was 0.31 ng/ml and the inter- and intra-assay coefficients of variation were <10%. sE-CAD was detectable in all tested specimens. To avoid selection bias for women who had multiple blood samples available, we averaged values per trimester.

We performed western blots of maternal serum as previously described [20]. Non-pathologic placental lysate (20 μg total protein/lane) was used as positive control. Blots were incubated with rabbit polyclonal anti–E-CAD antibody (1:1000; Cell Signaling Technology, Danvers, MA), mouse monoclonal anti–E-CAD N-terminus antibody (1:1000; Invitrogen Co, Camarillo, CA) and a mouse monoclonal anti–E-CAD C-terminus antibody (1:1000; BD Biosciences, Bedford, MA). Antibody specificity was confirmed by blotting with isotype non-immune IgG.

E-CAD immunoprecipitation was performed using the Dynabeads® Antibody Coupling Kit (Life Technologies, Grand Island, NY). Magnetic beads were coated overnight at 37 °C with N-terminus anti–E-CAD monoclonal antibody (5 μg antibody/mg of beads). The coated beads were washed and resuspended in 500 μl of buffer containing 100 μl maternal serum. After overnight incubation (40 °C) the beads were washed and the immunoprecipitation product eluted with 20 μl reducing Laemmli buffer and analyzed by Western blotting. Optical density of immunoreactive bands was determined using the NIH Image J software (http://rsbweb.nih.gov).

2.4. Histology and immunohistochemistry

A pathologist examined all hysterectomy samples, documenting the presence and degree of myometrial invasion. Full thickness biopsies were collected immediately after surgery from the site of placental insertion and the opposite uterine wall. Placental bed biopsies and placenta villous tissue of women (n = 6) with an uncomplicated pregnancy and elective term cesarean delivery were used for comparison. Placenta (n = 4) from women with previa were also evaluated. Tissues were fixed in formalin, embedded in paraffin and cut as serial sections at 5 μm thickness.

Tissues were stained with Masson-Trichrome (Richard-Allan Scientific, Kalamazoo, MI) and the following antibodies: polyclonal anti–E-CAD (1:200, Cell Signaling Technology) and anti–β-Catenin (1:500, BD Biosciences), and monoclonal anti-E-CAD C-terminus domain (1:100, BD Biosciences), anti–E-CAD N-terminus domain (1:100, Invitrogen), anti–cytokeratin-7 (1:100, Invitrogen), anti–vimentin (1:100, Invitrogen), anti–ADAM10 (1:200, R&D systems) and anti–presenilin-1 (1:100, Abcam Inc, Cambridge, MA). After 1 h incubation at room temperature with the appropriate biotinylated secondary antibody (1:600, Jackson Immunochemicals), detection was performed using avidin-biotin staining (Vectastain Elite ABC, Vector Laboratories) with Vector NovaRed chromogen and hematoxylin as counterstain. Negative control slides were incubated with appropriate rabbit or mouse isotype IgG. Histological scores were assigned by 2 independent blinded investigators, visualizing 3 different areas from each slide. The intensity of nuclear, cytoplasmic, membranous and extracellular staining was quantified for myometrial cells, villous cytotrophoblast, syncytiotrophoblast, interstitial EVT and vascular endothelium using the following categories: 0 (no staining), 1+ (minimum staining), 2+ (moderate staining) and 3+ (intense staining).

2.5. Statistical analysis

Data were tested for normality (Shapiro–Wilk test) and reported as median and IQR. Data were analyzed using Student's t-tests, Kruskal–Wallis one-way analysis of variance (ANOVA), two-way ANOVA and Chi-square tests. A $p < 0.05$ was considered significant. Data analysis was performed with SPSS Version 19.0 (SPSS, Chicago, IL), Sigma Stat, version 12.5 (RockWare, Golden, CO) and MedCalc (Broekstraat, Belgium).

3. Results

3.1. Demographic and clinical characteristics

In Supplementary Table 1 we present the demographic, clinical and outcome characteristics of the subjects enrolled in our study. Women with invasive placentation more often had a prior Cesarean section and hence a scarred uterus.

3.2. Circulating soluble E-cadherin by ELISA

First trimester [GA: 6–$13^{6/7}$ week] maternal circulatory levels of sE-CAD were not different from non-pregnant controls (Fig. 1A). We observed a significant decrease in sE-CAD in the 2nd trimester [GA: $14^{0/7}$–$27^{6/7}$ weeks] ($p = 0.005$), followed by an increase in the maternal circulatory levels in the 3rd trimester [$28^{0/7}$–$36^{6/7}$ weeks]. There was no significant difference in circulatory sE-CAD levels between women with normal placentation, invasive placentation, and previa alone (Fig. 1B, $p = 0.657$).

3.3. Immunoreactivity of maternal sE-CAD by Western blot and immunoprecipitation

Using a polyclonal N-terminus anti–E-CAD antibody, multiple bands were identified including the expected molecular weights of full length E-CAD (124 kDa) and sE-CAD (80 kDa) (Fig. 2A) [21]. The banding pattern was similar between women with invasive versus normal placentation. Given the polyclonal nature of the antibody, presence of multiple bands precluded the precise identification of serum sE-CAD. Thus, we employed immunoprecipitation with a monoclonal N-terminus E-CAD antibody. A single specific band (80 kDa) was identified in maternal serum as opposed the predominant band at 124 kDa in placental lysate (Fig. 2B). Among samples undergoing immunoprecipitation (n = 8), there was a direct correlation between the optical density of the 80 kDa band and the sE-CAD ELISA level ($r = 0.778$, $p = 0.023$). Similar with