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Villous trophoblast apoptosis is elevated and restricted to cytotrophoblasts in pregnancies complicated by preeclampsia, IUGR, or preeclampsia with IUGR

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ABSTRACT

Human placental villi are surfaced by an outer multinucleated syncytiotrophoblast and underlying mononucleated cytotrophoblasts. Conflicting data have attributed one, or the other, of these villous trophoblast phenotypes to undergo enhanced apoptosis in complicated pregnancies, compared to term, normotensive pregnancies. We use high-resolution confocal microscopy after co-staining for E-cadherin, as a trophoblast plasma membrane marker, and for the cleavage products of cytokeratin 18 and PARP1, as markers for caspase-mediated apoptosis, to distinguish between apoptotic cytotrophoblasts and apoptosis within the syncytiotrophoblast. We test the hypothesis that increased caspase-mediated apoptosis occurs in villi of placentas derived from pregnancies complicated by preeclampsia, intrauterine growth restriction (IUGR), or both. We find significantly elevated apoptosis in villous cytotrophoblasts from women with preeclampsia and/or IUGR, compared to term, normotensive pregnancies. Apoptosis of cytotrophoblasts in villi from complicated pregnancies appears to progress similarly to what we found previously for apoptotic cytotrophoblasts in villi from in term, normotensive pregnancies. Notably, caspase-mediated apoptosis was not detectable in regions with intact syncytiotrophoblast, suggesting strong repression of apoptosis in this trophoblast phenotype *in vivo*. We suggest that the elevated apoptosis in cytotrophoblasts in preeclampsia contributes to the placental dysfunction characteristic of this disorder. We also propose that repression of apoptosis in the syncytiotrophoblast is important to prevent apoptosis sweeping throughout the syncytium, which would result in widespread death of this essential interface for maternal-fetal exchange.

1. Introduction

Human placental villi are surfaced by the syncytiotrophoblast, a continuous layer of terminally differentiated, multinucleated epithelial cells. The syncytiotrophoblast is in direct contact with the maternal blood and is involved in the fetal-maternal transfer of gases, nutrients and wastes. Cytotrophoblasts, mononucleated epithelial cells, underlie the syncytiotrophoblast and reside upon a basement membrane that separates them from the villous stroma. Cytotrophoblasts can divide, differentiate, and fuse with the syncytiotrophoblast, allowing for growth of the syncytium during development and for reepithelization of damaged regions of villi.

Exogenous stimuli that create oxidative and nitrative stress influence placental development and contribute to placental injury. For example, the trophoblast bilayer is denuded in areas where fibrin-type fibrinoid marks sites of injury. These sites are reepithelialized by new syncytiotrophoblast, achieved by fusion of cytotrophoblasts with existing syncytium [1,2]. Oxidative and nitrative stress and placental maldevelopment or injury associate with preeclampsia, with or without intrauterine growth restriction (IUGR) [3–6]. Placental dysfunction commonly results from these maladies, yielding sub-optimal maternal and fetal outcomes in the process [3,7].

Apoptosis is a type of cell death that is often increased in response to stress and is regulated through an extrinsic pathway using cell-surface receptors, through an intrinsic pathway that involves mitochondrial depolarization, and by activation of the unfolded protein response of the ER [8,9]. Apoptosis is typically effected by activated caspase proteases that cleave substrates on the membranes, in the cytoplasm and nucleus, and in the fragmentation and degradation of nuclear DNA. Ultimately, an apoptotic cell undergoes fragmentation, forming multiple, vesicle-enclosed apoptotic bodies.

Abbreviations: clCyt18, cleaved cytokeratin 18; clPARP, cleaved poly-ADP-ribose polymerase; IUGR, intrauterine growth restriction.

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Immunolocalization of caspase-cleaved substrates and assays for fragmented nuclear DNA by TUNEL assays suggest that, compared to controls, there is an increased level of villous trophoblast apoptosis in pregnancies complicated by preeclampsia [10–12] or IUGR [13–15]. However, only few studies have attempted quantification, and these have reported large variations in the level of apoptosis, likely due in part to the differing methods used and their differing sensitivities and specificities [16,17].

An important, unresolved question is, what is the villous trophoblast phenotype that undergoes increased apoptosis in complicated pregnancies? Multiple previous studies, including our own, did not assign the apoptotic cells to a trophoblast phenotype [12,15,18,19]. Other studies concluded that the increased apoptosis was in localized regions of the syncytiotrophoblast [10,11,13,14,20,21], while others concluded apoptosis was higher in cytotrophoblasts of villi in complicated pregnancies, compared to control [16,22].

One likely contributor to these discrepant results is the difficulty in the definitive identification of villous cytotrophoblasts from the syncytiotrophoblast, because of their close and intertwined anatomical proximity [23,24]. Burton and colleagues [16] highlighted this problem in their electron microscopic study of villi from 6 to 15 weeks' gestation. They noted that cytotrophoblasts with morphological features of apoptosis lost contact with the basement membrane and were often interdigitated into, or even within, the syncytiotrophoblast cytoplasm. We recently found that both non-apoptotic and apoptotic cytotrophoblasts were frequently interdigitated within the syncytiotrophoblast of term villi, and that assignment of the trophoblast phenotype undergoing apoptosis required the use of high-resolution confocal microscopy and co-staining for E-cadherin, a marker of the trophoblast plasma membrane, and for markers of apoptosis [24]. We now apply this approach to test the hypothesis that increased caspase-mediated apoptosis occurs in villi of placentas derived from pregnancies complicated by preeclampsia, intrauterine growth restriction, or both. We further examine the premise that the increased apoptosis is in cytotrophoblasts and not in intact syncytiotrophoblast.

2. Materials and methods

2.1. Study participants, tissue procurement, fixation and sectioning

The Institutional Review Board of the Washington University School of Medicine approved this study. Placentas were obtained from singleton gestations with normotensive pregnancies and from pregnancies with preeclampsia, IUGR, or both. Preeclampsia was defined by the criteria of the American College of Obstetrics and Gynecology [25]. IUGR was defined as birth weight <10th percentile for the gestational age. The clinical characteristics of the patients in these groups are listed in Supplementary table 1, as recommended [26]. Within 20 min of delivery, systematic random sampling of each placenta yielded four tissue samples that were fixed in 10% neutral buffered formalin for 24 h, embedded in paraffin, and sectioned at 10 μm thickness.

2.2. Immunofluorescence and immunohistochemistry

We previously described methods for tissue deparaffinization, antigen retrieval, DNA staining with DRAQ5, and immunofluorescence detection of E-cadherin and for the caspase-cleaved forms of cytokeratin 18 (clCyt18) or poly-ADP-ribose polymerase (clPARP) by confocal microscopy with a Nikon Eclipse microscope equipped with a C1 laser scanning head and 60× objective [24], with typical image size of 1024 × 1024 pixels covering 212 μm × 212 μm with Z-stack steps of <0.5 μm. Images were optimized with ImageJ (rsbweb.nih.gov/ij/) using only linear adjustments. To avoid bias in image acquisition, focus was obtained using transmitted light on the upper left-hand portion on tissue sections of each slide, and a confocal Z-stack was acquired, followed by scoring for E-cadherin, DNA and clCyt18. The slide was moved down one field of view and the procedure repeated until >150 cytotrophoblasts had been captured. This was repeated for each of the four tissue samples from each placenta, resulting in >600 cytotrophoblasts (with ~6000 syncytiotrophoblast nuclei) being scored per placenta. Scoring of percent apoptosis using clCyt18 staining was as described [24]. Briefly, a region of E-cadherin staining with a diameter of >5 μm that outlined a region with clCyt18, with or without DNA, was scored as a single clCyt18-positive cytotrophoblast. Regions of E-cadherin staining of ≤5 μm diameter with clCyt18 were scored as derived from a single cytotrophoblast if all were within <50 μm of each other, and the percent of apoptotic cytotrophoblasts was calculated by dividing the total number of apoptotic cytotrophoblasts by the total number of apoptotic and non-apoptotic cytotrophoblasts and multiplying by 100. Samples were scored by an observer who was blinded to the clinical outcome of the patients until after all tissues had been scored.

For immunohistochemistry, after antigen retrieval tissues were incubated in 5% H_2O_2 in water at 23 °C for 5 min and washed with water for 5 min. Blocking, incubation with primary and secondary antibodies and antigen detection were done with the R.T.U. Vectastain Universal Elite kit with ImmPACT DAB substrate (Vector labs, Burlingame, CA), according to the manufacturers' recommendations. Nuclei were counterstained for 30 s at 23 °C using Hematoxylin QS (Vector Labs) and images obtained with Nikon E800 microscope with a color Olympus DT digital camera and a 60× objective.

Regions with fibrin-type fibrinoid [27] were identified by their characteristic hypocellular, eosinophilic appearance under light microscopy [2].

2.3. Statistical analyses

Comparisons for number of clCyt18-positive regions between term, normotensive and complicated pregnancies were done using the non-parametric Wilcoxon–Mann–Whitney *U* test (Kaleidograph, Synergy Software, Reading, PA) with $p \leq 0.05$ for significance.

3. Results

3.1. Study population

The demographics of the patient population are shown in Table 1.

3.2. Progression of caspase-mediated apoptosis in cytotrophoblasts in complicated pregnancies

Confocal microscopy using Z-stack image acquisition at <0.5 μm-thick optical sections allowed definitive distinction of the two villous trophoblast phenotypes when tissues are co-stained for E-cadherin, to identify trophoblast plasma membranes, and DNA, to identify nuclei [24]. Cytokeratin 18, an intermediate filament protein, is expressed in the cytoplasm of cytotrophoblasts and the syncytiotrophoblast but not by cells in the villous stroma [18]. To identify the products of activated caspases, we used the M30 antibody, which recognizes caspase-cleaved cytokeratin 18 (clCyt18) [28] and an antibody that recognizes nuclear

Table 1
Demographic characteristics of the study population.

	Term, normotensive ($n = 10$)	Preeclampsia ($n = 9$)	IUGR ($n = 5$)	Preeclampsia with IUGR ($n = 5$)
Mean age(±SD)	31.2(±5.6)	30.6(±6.1)	27.8(±5.8)	23.6(±4.6)[a]
Race				
White(%)	7(70%)	3(33.3%)	2(40%)	2(40%)
Black(%)	2(20%)	5(55.6%)	2(40%)	2(40%)
Hispanic(%)	0(0%)	0(0%)	0(0%)	0(0%)
Asian(%)	1(10%)	0(0%)	1(20%)	0(0%)
Other	0(0%)	1(11.1%)	0(0%)	1(20%)
Smoking(%)	1(10%)	1(11.1%)	1(20%)	1(20%)
Mean BMI(±SD)	29.8(±6.7)	33.3(±7.6)	27.0(±10.9)	31.4(±2.5)
Nulliparous(%)	6(60%)	4(44.4%)	3(60%)	3(60%)
Chronic hypertension(%)	1(10%)	4(44.4%)	0(0%)	1(20%)
Pre-gestational diabetes(%)	1(10%)	2(22.2%)	0(0%)	1(20%)
Mean gestational age at delivery (±SD)	38.9(±1.3)	35.3(±5.3)	37.5(±3.6)	29.8(±2.1)[a]
Mean birth weight (grams, ±SD)	3454(±594)	2595(±918)	2399(±109)	1200(±463)[b]

IUGR, intrauterine growth restriction. SD = standard deviation.
[a] $p < 0.05$, ANOVA, compared to term, normotensive.
[b] $p < 0.05$, ANOVA, compared with term, normotensive and preeclampsia.