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Reduced expression of the epidermal growth factor signaling system in preeclampsia

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ABSTRACT

Introduction: The epidermal growth factor (EGF) signaling system regulates trophoblast differentiation, and its disruption could contribute to perinatal disease. We hypothesized that this pathway is altered in preeclampsia, a disorder associated with trophoblast apoptosis and failure to invade and remodel the uterine spiral arteries.

Methods: Six EGF family peptides and a truncated EGF receptor splice variant (p110/EGFR) were examined using immunohistochemistry in the trophoblast of placentas (N = 76) from women with preeclampsia, and compared to placentas from women of similar gestational age (GA) with preterm labor (PTL) or small for gestational age (SGA) fetuses, as well as normal term placentas. EGF, transforming growth factor-α (TGFA), and heparin-binding EGF-like growth factor (HBEGF) were evaluated using ELISA in maternal plasma from another 20 pregnancies with or without preeclampsia. Cell death was evaluated in the HTR-8/SVneo human cytotrophoblast cell line using TUNEL to evaluate the protective effects of EGF peptides.

Results: Trophoblast HBEGF, TGFA, and EGF were significantly reduced in preeclampsia compared to PTL and SGA, while p110/EGFR accumulated significantly on the surface of the chorionic villi ($p < 0.05$). Plasma EGF levels were significantly decreased in preeclamptic patients, compared to non-preeclamptic patients ($p < 0.05$). HBEGF, EGF, TGFA, epiregulin, and betacellulin each blocked cytotrophoblast cell death *in vitro* ($p < 0.05$).

Discussion: Three members of the EGF family are dysregulated in placentas with preeclampsia, whereas p110/EGFR, a potential EGF receptor antagonist, is overexpressed. These findings are consistent with the concept that disruption of the EGF signaling system contributes to aberrant trophoblast development associated with preeclampsia.

Abbreviations: PE, preeclampsia; EGF, epidermal growth factor; EGFR, EGF receptor; TGFA, transforming growth factor-α; HBEGF, heparin-binding EGF-like growth factor; BTC, betacellulin; AREG, amphiregulin; EREG, epiregulin; p110/EGFR, 110 kDa truncated splice variant of the EGFR; SGA, small for gestational age infants; PTL, preterm labor; H/R, hypoxia/reoxygenation; KRT7, cytokeratin-7; TUNEL, terminal deoxunucleotidal transferase-mediated deoxyuridine 5-triphosphate nick end labeling; DAPI, 4′,6-diamidino-2-phenylindole.

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1. Introduction

Approximately 5% of human pregnancies are complicated by preeclampsia (PE), which is one of the leading causes of neonatal and maternal deaths in developed countries [1]. The syndrome is clinically defined by the presence of maternal hypertension and proteinuria occurring after 20 weeks of pregnancy in a previously normotensive, non-proteinuric patient [2]. Although the etiology of this condition is poorly understood, strong evidence supports involvement of deficient trophoblast survival, inadequate endovascular invasion, endothelial cell dysfunction and a systemic maternal inflammatory response [3–9]. Thus, events that occur during early placentation compromising trophoblast function could predispose to PE, while conditions arising much later in gestation exacerbate the onset of disease.

Accumulating evidence suggests that human trophoblast survival and invasive capacity are linked to intercellular signaling by peptides related to epidermal growth factor (EGF). EGF can protect against apoptosis induced during *in vitro* culture of human term cytotrophoblast cells [10,11], indicative of the ability of EGF and related proteins to act as survival factors. Peptide members of the EGF signaling system induce downstream signaling by binding to receptor trysosine kinases of the human EGF receptor (EGFR)/ERBB family, which contains four members [12,13]. Trophoblast motility and invasiveness are stimulated by EGF, transforming growth factor-α (TGFA) and heparin-binding EGF-like growth factor (HBEGF), based on *in vitro* studies of first trimester primary and immortalized cytotrophoblast cells [14,15]. HBEGF induction of extravillous trophoblast differentiation can be mediated by either EGFR/ERBB1 or ERBB4 [15]. HBEGF also protects first trimester cytotrophoblast cells from apoptosis when they are exposed to low concentrations of O_2 [16] or oxidative stress due to hypoxia/reoxygenation (H/R) injury [17]. Since preeclampsia is associated with failed trophoblast survival and invasive function [3,4,6], it is noteworthy that HBEGF expression is significantly reduced in the placentas of women with PE [18].

In addition to EGF, TGFA and HBEGF, the EGF family includes betacellulin (BTC), amphiregulin (AREG) and epiregulin (EREG) [12,13]. A truncated EGFR/ERBB1 isoform (p110/EGFR) has been identified and is elevated in PE patients [19]. Because p110/EGFR lacks the cytoplasmic domain, it could act as a dominant negative, adding to the complexity of this biochemical network.

Since the EGF signaling system includes several growth factors capable of regulating human trophoblast survival and invasiveness, including HBEGF, which is down-regulated in trophoblast cells from the placentas of women with PE, we hypothesized that members of the EGF family of peptides, in addition to HBEGF, and the p110/EGFR splice variant are dysregulated in placentas of women with PE. Similar outcomes in placentas can be found in conjunction with small for gestational age (SGA) infants. To compare for gestational age (GA), placentas from women with preterm labor (PTL) without evidence of PE, SGA, or infection were evaluated, and to compare for PTL, normal placentas delivered at term were evaluated. Because components of the EGF signaling system have been shown to reduce apoptosis in human trophoblast cells, EGF family members were compared for their ability to rescue trophoblast cells from apoptosis after H/R injury. Finally, we examined the hypothesis that EGF-like growth factors that are dysregulated by PE in the placenta are similarly altered in the circulation of patients with PE.

2. Materials and methods

2.1. Patient population

The Institutional Review Board of Wayne State University approved all consent forms and protocols used in this study, which abide by the NIH research guidelines. Placentas were obtained from pregnancies with: 1) PE (n = 35, mean GA = 31.8 weeks, SD = 3.8), defined as the presence of hypertension, proteinuria (+2) and delivery before the 35th week of gestation in nulliparous (N = 19) or multiparous (N = 16) women; 2) SGA infants without PE (N = 17, mean GA = 33.2 weeks, SD = 3.6), defined as a birth weight below the 10th percentile and without anomalies; and 3) spontaneous PTL leading to preterm delivery (N = 17, mean GA = 32.4 weeks, SD = 3.5) without histological chorioamnionitis (Table 1). Tissues from patients with PE, SGA, or PTL of similar GA (19–35 weeks) were compared at the time of delivery. There were no significant differences in GA among the three patient groups. Additional placentas were obtained from patients with uncomplicated pregnancies delivering at term (N = 7, mean GA = 39.2 weeks, SD = 0.3). The smaller N of the term group reflects the narrower range of GA compared to PTL. P110/EGFR staining was compared amongst PE (N = 29, mean GA = 31.3, SD = 3.2), SGA (N = 13, mean GA = 32.0, SD = 3.3), PTL (N = 10, mean GA = 31.4, SD = 3.9), term (N = 6, mean = 38.3, SD = 1.0). Placental tissues were selected from a large pool of archived tissues, based on patient inclusion criteria and GA matching.

In another group of patients, blood was collected at the time of diagnosis of PE (N = 20, mean GA = 33.6 weeks, SD = 3.71) and non-PE (N = 20 mean GA = 34.1 weeks, SD = 4.2) patients of similar GA.

2.2. ELISA

Patient blood plasma was separated by centrifugation at 1000 RPM for 10 min. ELISAs for HBEGF, EGF, and TGFA were preformed using DuoSet ELISA kits (R&D Systems, Minneapolis, MN), as previously described [16]. Standard curves were constructed for each assay and optical density of the final reaction products were determined at 450 nm using a programmable PowerWave (BioTek Instruments, Winooski, VT) microplate spectrophotometer with automatic wavelength correction.

2.3. Immunohistochemistry and quantification by image analysis

Formalin-fixed, paraffin-embedded tissue sections were labeled by immunohistochemistry as previously described [18,20]. Briefly, staining procedures were preformed using a DAKO autostainer universal staining system (Carpintera, CA). Slides were labeled with an antibody against p110/EGFR [21], an affinity-purified polyclonal antibody 1005 against the C-terminal domain of EGFR (Santa Cruz Biotechnology, Dallas, TX), or affinity purified polyclonal antibodies raised against the recombinant proteins, HBEGF, EGF, TGFA, BTC, AREG, and EREG (R&D Systems). Each primary antibody was titered using HTR-8/SVneo human cytotrophoblast cells grown on slides to ensure that labeling was linear with antibody concentration, as previously shown [20]. Primary antibody controls were preformed using non-immune IgG (Jackson Immunoresearch Laboratories, West Grove, PA). To assess the abundance of trophoblast cells in the tissues studied, adjacent sections were labeled with cytokeratin-7 (KRT7) using a monoclonal antibody (DAKO). Bound primary antibody was visualized using a peroxidase-conjugated polymer coupled to anti-rabbit and anti-mouse-IgG (EnVision Systems Peroxidase, DAKO). Slides were viewed under a DM IRB (Leica, Wetzlar, Germany) inverted microscope and brightfield images were obtained using an Orca (Hamamatsu, Hamamatsu City, Japan) digital camera or a Spot Jr. (Diagnostic Instruments, Inc., Sterling Heights, MI) color digital camera. Stain intensity was determined using simple PCI (Hamamatsu) image analysis software from monochrome images of three regions of each specimen, as detailed and validated elsewhere [20]. Average pixel densities are reported as grey level. Background grey level (non-immune IgG) was subtracted from each image to obtain the grey level values reported. Semi-quantification of p110/EGFR labeling was conducted using H scores. Three blinded observers assigned an H score [22] by subjectively scoring the staining intensity as 0, 1, 2, or 3. The three H scores were averaged for each image.

2.4. Cell culture and treatment

HTR-8/SVneo cytotrophoblast cells [23,24] were grown in 96 well plates and cultured in DMEM/F12 medium containing 10% fetal bovine serum. Prior to experimentation, medium was replaced with serum-free media containing 5 mg/ml bovine serum albumin. Control cells were cultured at 2% oxygen for 8 h in survival studies. H/R was modeled by culturing cells at 2% O_2 for 2 h, followed by culture at 20% O_2 for 6 h [17]. Cells were treated during the reoxygenation period by supplementing with medium pre-equilibrated at 20% with 0 nM, or 1 nM of EGF, TGFA, AREG, HBEGF, BTC, or EREG. Intravenous administration of $MgSO_4$ to patients with severe PE is the standard of care for seizure prophylaxis. Therefore, to determine the effect of $MgSO_4$ on HBEGF expression, cytotrophoblast cells cultured at 20% O_2 were treated with 0, 2, 5, and 10 mM $MgSO_4$ for 24 h, 72 h, or 7 days. Concentrations of 0.75–1.2 mM/L of $MgSO_4$ are equivalent to 1.5–2.4 mEq/liter blood concentration. The concentrations chosen in our experiment cover the therapeutic blood level concentrations of 3.5–7 mEq/liter we would anticipate to see in PE patients treated with $MgSO_4$ [25]. Cells were fixed with 4% paraformaldehyde at the conclusion of all *in vitro* experiments before subsequent analysis.