Published in final edited form as:

Placenta. 2015 March ; 36(3): 270–278. doi:10.1016/j.placenta.2014.12.006.

Reduced Expression of the Epidermal Growth Factor Signaling System in Preeclampsia

D. Randall Armant, PhD^{1,2,4}, Rani FRITZ, DO^{1,3}, Brian A. KILBURN, BS¹, Yeon Mee KIM, MD⁵, Jyh Kae NIEN, PhD⁵, Nita J. MAIHLE, PhD⁶, Roberto ROMERO, MD^{5,7,8}, and Richard E. LEACH, MD^{9,10}

¹Department of Obstetrics & Gynecology, Wayne State University School of Medicine, Detroit, MI

²Department of Anatomy & Cell Biology, Wayne State University School of Medicine, Detroit, MI

³Department of Physiology, Wayne State University School of Medicine, Detroit, MI

⁴Program in Reproductive and Adult Endocrinology, Division of Intramural Research, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD

⁵Perinatology Research Branch, Program for Perinatal Research and Obstetrics, Division of Intramural Research, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD

⁶Georgia Regents University Cancer Center, Augusta, GA

⁷Department of Obstetrics and Gynecology, University of Michigan, Ann Arbor, MI

⁸Department of Epidemiology and Biostatistics, Michigan State University, East Lansing, MI

⁹Department of Obstetrics, Gynecology & Reproductive Biology, College of Human Medicine, Michigan State University, Grand Rapids, MI

¹⁰Department of Obstetrics, Gynecology and Women's Health, Spectrum Health Medical Group, Grand Rapids, MI

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.

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Author for correspondence: D. Randall Armant, Ph.D., C.S. Mott Center for Human Growth and Development, Wayne State University School of Medicine, 275 E. Hancock Avenue, Detroit, MI 48201-1415, Tel.: (313) 577-1748; Fax: (313) 577-8554; D.Armant@Wayne.edu.

The authors report no conflict of interest.

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Methods—Six EGF family peptides and a truncated EGF receptor splice variant (p110/EGFR) were examined using immunocytochemistry 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.

Key Words/Phrases

preeclampsia; epidermal growth factors; placenta

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 tyrosine 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 O₂ [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 preeclampsia [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 downregulated 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.

MATERIALS AND METHODS

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. 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.

ELISA

Patient blood plasma was separated by centrifugation at 1000 RPM for 10 minutes. 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.

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 (Carpinteria, CA). Slides were labeled with an antibody against p110/EGFR [21] or affinity purified polyclonal antibodies raised against the recombinant proteins, HBEGF, EGF, TGFA, BTC, AREG, and EREG (R&D Systems). Each primary antibody was titrated 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.

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 hours in survival studies. H/R was modeled by culturing cells at 2% O₂ for 2 hours, followed by culture at 20% O₂ for 6 hours [17]. Cells were treated during the reoxygenation period by supplementing with medium pre-equilibrated at 20% with 0 nM, or 1nM 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 hours, 72 hours, or 7 days. Concentrations of 0.75–1.2 mM/liter 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.

TUNEL assay for cell death

Cell death was detected by terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick and labeling (TUNEL), using a fluorescein-based kit from Roche Applied Science (Indianapolis, IN). Cells were counterstained with 5 $\mu\text{g}/\text{ml}$ of 4',6-diamidino-2-phenylindole (DAPI). Fluorescent images were acquired with a Leica DM IRB epifluorescence microscope and an Orca digital camera. The TUNEL index was determined by calculating the percentage of TUNEL positive to DAPI labeled cells in triplicate fields in each well, as described previously [16, 17].

Statistical Analysis

SPSS (version 20.0) was used for statistical analysis. Stain intensities for proteins were compared among the four patient groups using Kruskal-Wallis analysis due to a non-normal distribution of the data. P110/EGFR staining intensities, blood levels, and demographic data were compared among the four patient groups using Steel-Dwass method due to non-normal distribution. One-way ANOVA was used to determine effects of MgSO_4 on HBEGF staining and H/R injury on TUNEL index. Spearman's rho was used to compare protein levels in the basal plate and chorionic villi to GA for PTL, PE, and SGA groups.

RESULTS

EGF-like growth factors in human placentas

It was previously reported that HBEGF is expressed in KRT7-positive villous and extravillous trophoblast cells [18]. Preliminary studies demonstrated no reactivity to antibodies against neuregulin/herregulin in human placental tissues above control labeling with non-immune IgG (data not shown); however, other members of the EGF family were expressed. The cellular distributions of EGF, TGFA, HBEGF, AREG, EREG and BTC were similar in the placentas of women delivering preterm or at term (data not shown), and each antigen was detected above the non-immune IgG controls (Fig. 1). However, there were relatively high levels of each growth factor in the villous trophoblast layer and in extravillous trophoblast cells invading the basal plate (arrows in Fig. 1).

Although HBEGF expression appeared to be strongest in trophoblast cells, staining in excess of the non-immune IgG control levels was present throughout the placental tissue (Fig. 1). To gain insight into which cells produce HBEGF, tissue sections were treated with heparitinase prior to immunohistochemistry to eliminate peripherally bound, secreted HBEGF. After heparitinase treatment, HBEGF was predominantly found in cytotrophoblast

cells of the chorionic villi and in putative extravillous trophoblast cells at the basal plate (Fig. 1), suggesting the cellular source of this growth factor.

EGF-like growth factors and p110/EGFR in normal and pathological placentas

Six EGF family growth factors were assessed in placentas from normotensive (N=41) and hypertensive (N=35) women delivering between 23 and 40 weeks of gestation. The patients were further subdivided into four groups: 1) normotensive women delivering at term (N=7); 2) patients with PE (N=35); 3) patients of similar GA at delivery with women with PTL (N=17); and 4) women delivering SGA infants (N=17). No differences in growth factor levels were found between placentas of multiparous (19) and nulliparous (16) women with PE (not shown), so all data for PE women were combined in the analysis of semi-quantitative data.

Demographic patient data are represented in table 1. Median GA levels were similar in the PE, SGA, and PTL, and were 32.6, 32.9, 32.9, respectively. Significant differences were found in the highest systolic and diastolic blood pressure values and proteinuria levels between the PE group compared to SGA, PTL, and term groups.

Median staining intensity values of each growth factor in the basal plate or chorionic villi are presented in Fig. 2. HBEGF was significantly reduced in both the chorionic villi and basal plate of placentas from pregnancies with PE, compared to term, PTL, and SGA groups. Median TGFA staining demonstrated a similar statistically significant down-regulation in the basal plate and chorionic villi of placentas from women with PE compared to all other groups. The other statistically significant difference in TGFA was found in the chorionic villi between term placentas and those of SGA fetuses. Expression of EGF in the basal plate of placentas from patients with PE was significantly reduced compared to all other patient groups. Higher EGF staining was detected in the chorionic villi of term placentas, differing significantly from SGA and PE placentas, but not from PTL. There were no significant correlations between GA and expression of HBEGF and TGFA in the basal plate and chorionic villi in any groups. For EGF, there was a significant increase seen with GA in the basal plate ($P=0.029$) and chorionic villi ($P=0.006$) of the PE group only (Supplementary data, Fig. S1).

The reduced expression of these growth factors in PE placentas could be the result of a decrease in the trophoblast cells due to apoptosis or failed invasion of the basal plate. However, the distribution of trophoblast populations within the placenta demonstrated by immunohistochemical labeling of KRT7 (Fig. 1) was not quantitatively different (ANOVA; $p=0.08$ in villi; $p=0.10$ in basal plate) among the PTL, SGA and PE groups in the basal plate or chorionic villi (Supplementary data, Table S1). Due to the fact that most patients with PE are treated with $MgSO_4$ for seizure prophylaxis, it is important to understand whether HBEGF expression is altered by $MgSO_4$ in trophoblast cells. Culturing HTR-8/SVneo human cytotrophoblast cells with $MgSO_4$ at doses of 2 to 10 mM for 1 to 7 days did not significantly alter HBEGF expression (Supplementary data, Table S2).

In contrast to EGF, TGFA and HBEGF, the median staining intensities of BTC, AREG and EREG were not consistently down-regulated in placentas from women with PE, as

compared to the other patient groups (Fig. 2). BTC was significantly elevated in the chorionic villi at term compared to all other groups and in the basal plate compared to SGA and PE. In chorionic villi, AREG was significantly elevated in term placentas compared to preterm labor and preeclampsia, and in the basal plate compared to preterm labor. EREG demonstrated a significant increase with SGA as compared to PE and PTL in chorionic villi and as compared to PE in the basal plate.

The EGFR splice variant p110/EGFR was strongly expressed on the surface of chorionic villi of placentas at term, but not placentas delivered preterm (Supplementary data, Fig. S2). In placentas from patients with PE that were not at term, p110/EGFR was also highly expressed. However, antibody against full-length ERBB1/EGFR strongly labeled placentas of both the preterm labor and PE groups (Fig. S2), a result that was distinct from p110/EGFR. Because P110/EGFR was confined to the periphery of the villi, it was not compatible with the image analysis method used for EGF family growth factors. Therefore, staining of P110/EGFR was semi-quantitative by H score, which confirmed significant elevation in PE, as compared to SGA and PTL patients, however, P110/EGFR levels in term placentas were comparable to PE patients (Table 2).

EGF-like growth factors are cytoprotective for trophoblast

Previously, we found that apoptosis is low and not significantly different when human first trimester trophoblast cells are cultured at either 20% or 2% O₂, but increases significantly after switching from low to high O₂ [17]. In an H/R model using the HTR-8/SVneo cytotrophoblast cell line, cell death due to H/R injury was blocked by supplementation with the EGF-like growth factors, HBEGF, EGF, TGFA, EREG or BTC (Fig. 3). Cell death was not significantly reduced by AREG, although there was a downward trend.

Plasma EGF Levels

Circulating EGF, TGFA and HBEGF were examined preterm in a separate group of women who either were diagnosed with PE or were normotensive and later delivered at term (Table 1). The average age of the patients in the normotensive (26 ± 4.6) and PE (24 ± 7.7) groups and the GA at time of blood draw in the normotensive (33.6 ± 3.7) and PE (34.1 ± 4.1) groups were not significantly different. The median blood plasma concentrations of HBEGF and TGFA in PE patients were not significantly different from normotensive control patients (Figure 4). However, median control plasma EGF levels (324.3 pg/ml) were more than double ($p < 0.05$) the median concentration (147.5 pg/ml) in PE patients (Figure 4). Regression analysis indicated no effect of GA on EGF plasma levels in either the control ($R^2=0.0040$) or PE ($R^2=0.0055$) group.

DISCUSSION

We report, for the first time, evidence for broad down-regulation of the EGF signaling system in trophoblast cells of placentas from PE pregnancies. In agreement with our previous report [18], it was demonstrated that HBEGF protein levels are significantly decreased in PE placentas as compared to term, PTL, and SGA placentas. HBEGF dysregulation could play a critical role in the etiology of PE, particularly if other

components of the EGF signaling system contribute to suppression of signaling. Indeed, TGFA and EGF were also significantly lower in placentas from women with PE, further supporting the hypothesis that deficiencies of the EGF signaling system in PE contribute to improper extravillous trophoblast differentiation and increased apoptosis. Since EGF, TGFA and HBEGF are each capable of altering integrin expression associated with increased motility and invasiveness of human trophoblast cells [15], their deficiency could contribute to uteroplacental insufficiency. Others have shown that TGFA promotes cytotrophoblast proliferation [26] and that EGF promotes trophoblast invasion [14]. Furthermore, the EGF signaling system can limit trophoblast apoptosis, as demonstrated during H/R injury of cultured cytotrophoblast cells and here elsewhere [10, 11, 16, 17, 27–29]. The reason for dysregulation of EGF family growth factors in PE is not clear; however, a genetic predisposition is suggested by polymorphisms in the HBEGF gene associated with the development of PE [30]. There were no differences found in expression of EREG between PE and term or PTL. Although AREG and BTC were significantly altered in PE placentas compared to term, they were comparable to GA-matched PTL and SGA patients of similar GA, suggesting a GA effect.

Although some EGF-like growth factors are expressed normally in PE, it is not known whether their activity is sufficient for trophoblast survival and invasion *in vivo*. BTC and EREG were as effective as HBEGF, EGF and TGFA in blocking H/R induction of apoptosis in cultured cytotrophoblast cells. However, the function of residual EGF-like growth factor expression in PE could be suppressed by truncated ERBB splice variants that act as antagonists or dominant negative. One such protein shown here to be highly expressed in placentas of PE women is p110/EGFR, a 110-kDa isoform of the EGF receptor thought to contain a glycosylphosphatidylinositol anchor [31]. Indeed, p110/EGFR was previously isolated from human placentas [31]. There are other truncated isoforms of the EGFR produced by alternate mRNA splicing or proteolytic cleavage of the full-length receptor [32]. Evaluation of EGFR mRNA expression in PE placentas revealed significantly higher levels of the truncated transcript [19]. Consistent with those findings, we found an increase in p110/EGFR in placentas of PE patients, compared to SGA and PTL patients. Given that p110/EGFR contains an extracellular domain and lacks an intracellular cytoplasmic domain, it should provide no intracellular signaling upon EGF binding and act as a dominant negative. The high levels of p110/EGFR found in PE placentas could, therefore, exacerbate the paucity of EGF family growth factors. p110/EGFR also increased in term placentas, confirming a previous report that EGFR rises significantly at term [33]. The increase in P110/EGFR at term suggests a need to downregulate the EGF signaling system at term, as persistent trophoblast invasion is noted in patients with post-partum hemorrhaging due to subinvolution of the utero-placental arteries [34]. Further studies are required to evaluate the precise function and clinical relevance of this EGF receptor isoform as it pertains to PE and parturition.

Patients with severe PE, similar to the PE cohort studied here, are routinely treated with MgSO₄ for seizure prophylaxis, as is the standard of care. To ensure that the decrease in HBEGF found in PE placentas was not an effect of MgSO₄ administration, cytotrophoblast cells were cultured in MgSO₄ for up to 7 days. No significant dose-dependent decrease in

HBEGF was found after MgSO_4 treatment *in vitro*, suggesting that MgSO_4 is not responsible for the low levels of HBEGF in PE placentas. Furthermore, the decreased expression of HBEGF, TGFA and EGF in PE placentas could not be explained by a significant reduction of trophoblast cells populating the chorionic villi or basal plate, based on comparable expression of the trophoblast marker protein KRT7 in all patient groups.

It is well-established that placentas are subject to numerous forms of stress during pregnancy, including oxidative and inflammatory stress [9, 35]. PE placentas have increased rates of apoptosis, presumably related to oxygenation injury [3, 4]. Injury could be caused by oxygen radicals produced by abnormally high levels of oxygen during placentation [36]. Previously, we reported that survival of human first trimester cytotrophoblast cells at the low O_2 concentrations experienced by the conceptus during the first 10 weeks of pregnancy [37, 38] correlates with a pronounced up-regulation of HBEGF [16]. Here, we show that HBEGF, EGF, TGFA, BTC, and EREG can each prevent cell death in a cytotrophoblast cell line due to oxidative stress caused by H/R. We have previously found that exogenous application of HBEGF to cytotrophoblast cultures will prevent apoptosis caused by experimentally-induced oxidative stress and that the effect is dependant on the ERBB receptor mediated downstream signaling [16, 17, 28]. These findings are consistent with previous studies that have shown that EGF reduces apoptosis in trophoblast cells [39].

Because EGF, HBEGF, and TGFA are decreased in placental tissues from PE, we examined the possibility that their blood levels are altered in PE patients. Plasma EGF levels were significantly decreased in patients with PE compared to those without the disease, while HBEGF and TGFA were unaffected. The absence of a difference in TGFA between preeclamptic and non-preeclamptic patients could reflect the very low levels of TGFA detected in serum. HBEGF is secreted by multiple organs [40], possibly overshadowing the contribution from the placenta. Furthermore, heparan sulfate expressed on the trophoblast surface might retain HBEGF produced in the placenta. The decrease in circulating EGF of PE patients is consistent with the decreased expression by the placenta. Previous studies evaluating EGF blood levels in IUGR, which shares similar placental pathophysiology with PE [41], have yielded inconsistent results. Similar to our study, an evaluation of EGF plasma levels in IUGR patients found significantly lower levels compared to non-IUGR patients [42], while another study evaluating blood EGF levels in IUGR patients found no difference [43]. Our findings suggest that EGF blood levels are dysregulated in patients with PE. It would be important to further assess EGF blood levels prior to the clinical onset of PE and follow these patients to term. If dysregulated EGF blood levels predate the placental phenotype seen at the time of diagnosis of PE, then dysregulation of EGF could contribute to placental disease. Furthermore, EGF blood levels might be useful as a pre-clinical biomarker.

We have presented new information reporting that several members of the EGF family are decreased in the placentas patients with PE, suggesting a role in its etiology. This study evaluated placentas at delivery; however, disruption of the EGF signaling system likely occurs early in pregnancy. It is not possible to study first trimester placentas in PE patients because the disease is not diagnosed until at least 20 weeks of gestation. The utility of this

approach awaits a non-invasive and safe method of evaluating trophoblast expression early in pregnancy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant numbers and sources of support:

This work was supported, in part, by the Intramural Research Program of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, (NICHD/NIH) Department of Health & Human Services; and by the NICHD/NIH through cooperative agreement U54HD40093 as part of the Specialized Cooperative Centers Program in Reproduction and Infertility Research.

We wish to thank Dr. Anelia Petkova and Ms Stella Dewar for their expert technical assistance. We would also like to thank Dr. Hamid-Reza Kohan-Ghadr for his help on statistical analysis.

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 EGF receptor
SGA	Small for gestational age infants
PTL	Preterm labor
H/R	hypoxia/reoxygenation
KRT7	cytokeratin-7
TUNEL	Terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick and labeling
DAPI	4',6-diamidino-2-phenylindole

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Highlights

- EGF, HBEGF, and TGFA are significantly decreased in placentas from preeclamptic patients.
- Expression of EGFR splice variant p110/EGFR is significantly increased in placentas from preeclamptic patients.
- EGF growth factors are protective against hypoxia/reoxygenation injury in cytotrophoblast cells.
- Plasma EGF levels are significantly decreased in preeclamptic patients.

Figure 1. Localization of EGF family proteins in normotensive placentas

Paraffin-embedded sections of human placental tissues were labeled by immunohistochemistry with primary antibodies against KRT7 (A), EGF (B), TGFA (C), HBEGF (D), BTC (F), AREG (G), EREG (H), and non-immune IgG (E). Arrows indicate putative extravillous trophoblast cells in the basal plate regions, based on KRT7 labeling patterns in adjacent sections. I-N. Immunohistochemistry for HBEGF before (I, L) and after (J, K, M, N) sections were treated with heparitinase to eliminate peripherally bound, secreted HBEGF. The latter sections reveal the cellular origin of HBEGF in the basal plate (J, K) and chorionic villi (M, N). Higher magnification images of basal plate and chorionic villi are shown in panels K and N, respectively. Size bars indicating 50 μ m are shown in panels E, M and N. The size bar in panel E corresponds to A through H. The size bar in panel M corresponds to panels I, J, L, and M, whereas the size bar in panel N corresponds to N and K.

Figure 2. Quantification of growth factor levels in placental tissues

Immunohistochemical labeling of the indicated growth factors was quantified by image analysis of the basal plate (BP) or chorionic villous (Villi) regions of placental sections from pregnancies with preterm labor (PTL), small for gestational age infants (SGA), preeclampsia (PE) or term delivery (TERM). The y-axis is the grey level in arbitrary units after subtracting the value for non-immune IgG controls. The thick horizontal line indicates a value of 0. *, $p < 0.05$ compared to all other groups. In other panels, a,b,c, groups with the same lower case letters are not different, whereas groups with no letters in common, $p < 0.05$.

Figure 3. Prevention of apoptosis due to H/R by EGF-like growth factors

Experiment was run in triplicates. HTR-8/SVneo cytotrophoblast cells were cultured at 2% O₂ (Hypoxia) or exposed to 2% O₂ for 2 hours followed by 6 hours at 20% O₂ (H/R). In other treatments, culture medium was supplemented with 1 nM of the indicated growth factors during H/R. Apoptosis was assessed by TUNEL assay. *, p<0.05 compared to Hypoxia.

Figure 4. Serum Growth Factor Concentrations in Non-preeclamptic and Preeclamptic Patients
 Box plot represents comparisons of EGF, HBEGF, and TGFA blood levels. Values are represented as median with inter-quartile range. * denotes outlying values. Significant decrease ($P < 0.05$) was found for EGF levels in the PE group compared to the non-PE group.

Table 1

	PE	SGA	PTL	Term
Number, <i>n</i> (% of total)	35 (46)	17(22.3)	17(22.3)	7(9.4)
Maternal age in years, <i>median (IQR)</i>	26 (21 – 35)	23 (19.75 – 26.25)	21 (20 – 26)	24 (22 – 30)
Smoking habit				
Smoker, <i>n</i> (%)	7 (20)	4 (24)	5 (29)	2 (29)
Non-smoker, <i>n</i> (%)	28 (80)	13 (76)	12 (71)	5 (71)
Ethnicity				
Caucasian, <i>n</i> (%)	8 (23)	1 (6)	2 (12)	1 (14)
African, <i>n</i> (%)	25 (71)	15 (88)	13 (76)	6 (86)
Asian, <i>n</i> (%)	1 (3)	0 (0)	0 (0)	0 (0)
Hispanic, <i>n</i> (%)	1 (3)	1 (6)	1 (6)	0 (0)
Unknown, <i>n</i> (%)	0 (0)	0 (0)	1 (6)	0 (0)
Gestational age at delivery in weeks, <i>median (IQR)</i>	32.6 (29.6 – 34.6)	32.9 (30.7 – 35.5)	32.9 (28.6 – 33.7)	39 (39 – 39)
Gravity, <i>mean (SEM)</i>	2 (1 – 3.5)	2.5 (2 – 4)	2 (1 – 3)	5 (3.5 – 5.5) *
Parity, <i>mean (SEM)</i>	0 (0 – 1)	1 (0 – 2.25)	0 (0 – 1)	2 (1.5 – 2) *
Delivery mode				
Normal Spontaneous Vaginal Delivery (NSVD), <i>n</i> (%)	7 (20)	8 (47)	11 (65)	0 (0)
Cesarean section (CS), <i>n</i> (%)	28 (80)	8 (47)	6 (35)	7 (100)
Unknown, <i>n</i> (%)	0 (0)	1 (6)	0 (0)	0 (0)
Birth Weight (gr), <i>median (IQR)</i>	1460 (900 – 1800)	1410 (850 – 1690)	2080 (998.7 – 2400)	3110 (2820 – 3560) *
Sex				
Male, <i>n</i> (%)	20 (57)	7 (41)	11 (65)	5 (71)
Female, <i>n</i> (%)	15 (43)	10 (59)	6 (35)	2 (29)
Systolic blood pressure (mmHg), <i>median (IQR)</i>	172 (160 – 181.5)	147 (136 – 155.5) *	120 (120 – 132) *	<i>N/R</i>
Diastolic blood pressure (mmHg), <i>median (IQR)</i>	109 (101.5 – 119.5)	83 (75 – 89) *	80 (74 – 80) *	<i>N/R</i>
Proteinuria (mg/dl), <i>median (IQR)</i>	3 (2 – 3)	0 (0 – 0.5) *	0 (0 – 0) *	<i>N/R</i>
APGAR score (1 min), <i>median (IQR)</i>	8 (5 – 8)	8 (6.75 – 8)	8 (5 – 8)	8 (6.5 – 8.5)
APGAR score (5 min), <i>median (IQR)</i>	9 (7 – 9)	9 (8 – 9)	9 (9 – 9)	9 (9 – 9)

Table 2

P110/EGFR Expression in Chorionic villi of placentas from women with PTL, SGA, PE, and healthy term pregnancies.

Groups	Median (IQR)	Significance *
PTL	0 (0–0)	a
SGA	0 (0–0.33)	a
Term	1.83 (1.25–3)	b
PE	2.67 (0.33–3)	b

H scores were determined by the average of three blinded observers subjective readings between 0 and 3 for PE (N= 29), PTL (N= 10), SGA (N= 13), and term (N= 6) to each labeled placental section to indicate the level of p110/EGFR immunohistochemical staining. Labeling was predominantly on the surface of the chorionic villi. The readings were averaged and analyzed using non-parametric statistics. Medians and inter-quartile ranges (IQR) are shown.

* Groups labeled with a and b were significantly different ($p < 0.05$) according to Steel-Dwass Method.