Expression of Heparin-binding EGF-like Growth Factor in Term Chorionic Villous Explants and Its Role in Trophoblast Survival


ABSTRACT

Heparin-binding EGF-like growth factor (HBEGF) induces trophoblast extravillous differentiation and prevents apoptosis. These functions are compromised in preeclampsia. Because HBEGF is downregulated in placentas delivered by women with preeclampsia, we have examined its expression and cytoprotective activity in term villous explants. Chorionic villous explants prepared from non-pathological placentas collected by cesarean section at term were cultured at either 20% or 2% O2 and treated with the HBEGF antagonist CRM197 or recombinant HBEGF. Paraffin sections were assayed for trophoblast death, proliferation and HBEGF expression using the TUNEL method, immunohistochemistry for nuclear Ki67 expression and semi-quantitative immunohistochemistry with image analysis, respectively. Trophoblast cell death was increased significantly after 8 h of culture with CRM197 or by culture for 2 h at 2% O2. Exogenous HBEGF prevented cell death due to hypoxia. Proliferative capacity was not affected by culture at either 20% or 2% O2. Contrary to first trimester placenta, term trophoblasts do not elevate HBEGF expression in response to hypoxia. However, low endogenous levels of HBEGF are required to maintain survival. Therefore, HBEGF-mediated signaling significantly reduces trophoblast cell death at term and its deficiency in preeclampsia could negatively impact trophoblast survival.

1. Introduction

Preeclampsia is a leading cause of maternal and perinatal morbidity and mortality worldwide. Although the causes of this syndrome are poorly understood, strong evidence lends support to roles for abnormal trophoblast invasion, endothelial cell dysfunction and a systemic maternal inflammatory response [1,2]. Increased rates of apoptosis have been reported in placentas from preeclamptic pregnancies [3,4]. A failure of physiological transformation of the spiral arteries by cytotrophoblast cells, which is associated with aberrant expression of adhesion molecules [5], has also been noted in preeclampsia. Additionally, restriction of trophoblast invasion [6] and survival [7] occurs in conjunction with the birth of small for gestational age (SGA) infants, suggesting shared elements in the etiology of both disorders.

An important ligand in the epidermal growth factor (EGF) signaling system, heparin-binding EGF-like growth factor (HBEGF), accumulates in both villous and extravillous trophoblasts and is expressed at high levels in the first trimester and throughout gestation in normal pregnancies [8,9]. HBEGF induces an invasive trophoblast phenotype in human [10] and mouse [11] blastocysts, and can initiate molecular and cellular changes characteristic of decidualization in a pseudopregnant mouse uterus [12]. Moreover, HBEGF is a survival factor that inhibits apoptosis [13]. Apoptosis induced by either transforming growth factor-β or tumor necrosis factor-α in human endometrial stromal cells is reduced by HBEGF [14]. These functions of HBEGF during early gestation could persist in trophoblasts to sustain survival throughout pregnancy. Indeed, exogenous application of EGF has been shown to reduce cell death in human term trophoblast cells subjected to oxidative stress or pro-apoptotic cytokines [15–18] more effectively than other growth factor ligands of receptor tyrosine kinases [17,19]. We have shown in a first trimester human cytotrophoblast cell line that their survival at 2% O2 is dependent on endogenous HBEGF signaling [20]. HBEGF, but not other EGF-like growth factors, increases within 4 h after exposure to 2% O2 and interference with HBEGF signaling both prevents its upregulation and increases apoptosis. HBEGF is optimally cytoprotective at 1 nM, which is approximately the concentration of medium conditioned by first trimester trophoblast cells cultured at 2% O2. It is not known whether this survival mechanism is operative after the first trimester. Overall, these
finding suggest that pathologial interference with HBEGF signaling could jeopardize trophoblast survival and diminish invasive activity during placentaion.

Although HBEGF can be detected in human placental tissues throughout gestation, its expression is reduced in pregnancies with SGA infants and is nearly undetectable in most pregnancies diagnosed with preeclampsia [21]. It is not clear how early during gestation the expression of this protein becomes dysregulated in women who are eventually diagnosed with preeclampsia or whether its loss contributes to the pathophysiology of the disease. However, its putative roles in trophoblast invasion and survival, which are both insufficient in preeclampsia and intrauterine growth restriction, suggest a relationship. Furthermore, its function and regulation during mid to late gestation requires clarification, since that is the period when pathological downregulation has been documented. In this study, we aimed to establish the regulation of HBEGF by O2 and its role in reducing cell death and preserving cell proliferation in term villous explants during in vitro culture and hypoxic stress. Our findings support its putative survival function and indicate the potential impact of its loss in pathologic placentas.

2. Materials and methods

2.1. Participants

Placentas (n = 5) were obtained by elective cesarean sections at term from patients who had non-pathologic pregnancies. Participants provided written informed consent; our protocol was approved by the Wayne State University Human Investigation Committee.

2.2. Villous explant culture

Several cotyledons from the central region of the placenta were removed and rinsed in cold sterile PBS to remove blood, and then dissected to remove the decidua and blood vessels. The chorionic villi were cut with scissors into pieces of approximately 5 mg wet weight, which were placed individually onto polycarbonate filter inserts in a 24-well culture plate (Costar, Corning, NY). The bottom chamber contained 500 μl DMEM/F12 (1:1) medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, penicillin and streptomycin, while the upper chamber contained approximately 25 μl of this medium. Villous explants were cultured at 37 °C in a humidified incubator containing 5% CO2 and air (20% O2) overnight before initiating the experiments. Experiments commenced with the addition of 150 μl of medium to the tissue in the upper chamber, with or without supplementation with 10 μg/ml CRM197 (EMD Chemicals, Inc., La Jolla, CA) or 1 nM recombinant HBEGF (R and D Systems, Minneapolis, MN), and the culture was continued for 0, 2, 8 or 24 h. Another set of villous explants was cultured in plates placed into plastic bags; both the lower and upper chambers were bubbled with air. These conditions were maintained in an incubator in which the atmosphere was 20% or 2% O2.

2.3. Immunohistochemistry for HBEGF

Immunohistochemistry was performed using a DAKO (Carpinteria, CA) Autostainer Universal Staining System, as previously described [21]. Rehydrated sections of cultured explants were labeled for 1 h at 25 °C with 5 μg/ml goat polyclonal antibody against human recombinant HBEGF (R and D Systems, Minneapolis, MN), and the culture was continued for 0, 2, 8 or 24 h. Another set of villous explants was cultured in plates placed into plastic bags; both the lower and upper chambers were bubbled with air. These conditions were maintained in an incubator in which the atmosphere was 20% or 2% O2.

2.4. Cell death and proliferation assays

Cell death was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL), using an alkaline phosphatase-based kit from Roche Applied Science (Indianapolis, IN) and counterstaining with hematoxylin. Slides were viewed at 200× magnification and the digital images were analyzed to determine the percentage of TUNEL/hematoxylin-labeled nuclei (TUNEL index) from triplicate fields. Sensitivity was assessed by immunochemistry with a mouse monoclonal antibody against Ki67 (DAKO) were counterstained with hematoxylin and similarly assessed for the percentage of Ki67/hematoxylin-labeled nuclei as an index of cell proliferation. 

2.5. Statistical analysis

Assays were conducted using triplicate samples and all experiments were repeated using five different placentas. Data were analyzed using the SPSS version 12.0 (SPSS, Chicago, IL) statistics program. For the effects of hypoxia and HBEGF on TUNEL and Ki67 expression, separate one-way ANOVAs were performed at each time point with the inclusion of the control (0 h). Where significance was found, a Student–Newman–Keuls post hoc test was used to determine differences among the treatments. One-way ANOVAs followed by Student–Newman–Keuls post hoc tests were used to analyze the effects of CRM197 on cell death and proliferation, and hypoxia on HBEGF levels. Histograms present the data as mean ± SE.

3. Results

Chorionic villi were collected after treatment for 0–24 h and evaluated for trophoblast cell death and proliferative capacity by determining the TUNEL and nuclear Ki67 expression, respectively. Examples shown in Fig. 1 suggest low levels of TUNEL during culture at 20% O2 (Panel A), but a strikingly higher number of TUNEL-positive trophoblast nuclei after only 2 h of culture at 2% O2 (Panel B). TUNEL-positive trophoblast cells were abundant after 24 h of hypoxic culture (Panel C), but not if the medium was supplemented with recombinant HBEGF (Panel D), suggesting a cytoprotective effect of the growth factor. Quantitative assessment of tissue from the five placentas cultured for 0–24 h confirmed these observations (Fig. 2A), demonstrating a significantly higher TUNEL index during culture at 2% than 20% O2 and amelioration of cell death when the hypoxic medium was supplemented with 1 nM HBEGF. Exogenous HBEGF had no effect on the low cell death levels during culture at 20% O2. Villous trophoblast cells from healthy, normotensive pregnancies contain endogenous HBEGF, but HBEGF is nearly absent in preeclampsia [21]. Therefore, we examined the effect of inhibiting endogenous HBEGF signaling during culture at 20% O2 by exposure to the HBEGF antagonist CRM197, a non-toxic mutant of diphtheria toxin that specifically binds to HBEGF and prevents interaction with its receptors, HER1 and HER4 [22]. Significant elevation of the TUNEL index was observed at 8 and 24 h (Fig. 2B), suggesting that endogenous HBEGF signaling is required for trophoblast survival during in vitro culture.

Trophoblast cell proliferation was not strongly affected by hypoxia or HBEGF. Proliferation rates rose slightly, but not significantly, at 2% O2 and were unchanged by supplementation with recombinant HBEGF (Fig. 3A). Inhibition of endogenous HBEGF signaling with CRM197 during culture at 20% O2 inhibited proliferation transiently at 2 h, but the nuclear Ki67 expression recovered by 8 h (Fig. 3B).

We previously demonstrated that first trimester human trophoblast cells rely on elevated HBEGF signaling to survive at 2% O2 [20]. To determine whether this mechanism persists in trophoblasts at term, HBEGF was localized by immunohistochemistry in villous explants. The HBEGF antibody stained the trophoblast and mesoderm at levels clearly above the background staining of non-immune IgG (Fig. 4A–D). Explants cultured for 24 h at either 20% or 2% O2 appeared to express HBEGF at levels similar to the non-cultured control villi. Semiquantitative analysis of HBEGF by immunohistochemistry revealed no difference in the levels of HBEGF during culture for 24 h at either 2% or 20% O2.
Unlike first trimester human cytotrophoblast cells [20], trophoblast at term does not upregulate HBEGF in response to hypoxic stress.

4. Discussion

Our findings demonstrate that HBEGF contributes significantly to the survival of term trophoblast cells during stress induced by in vitro culture and that it can prevent apoptosis during exposure to hypoxia. Elimination of endogenous HBEGF signaling during villous explant culture using the antagonist CRM197 significantly increased cell death among villous trophoblast cells. Although HBEGF levels in the placenta were relatively low, they provided significant protection against the cytological damage incurred during culture over a period of 8 h. More severe stress generated by culturing explants at 2% O₂ increased the TUNEL index within 2 h from 15–20% to nearly 80%. Supplementation with a cytoprotective concentration of HBEGF [20] during hypoxic culture blocked the dramatic increase in cell death. Poor survival at 2% O₂ suggested that term trophoblast cells lack the ability found in first trimester cytotrophoblast cells to elevate HBEGF during exposure to low O₂ concentrations [20]. Indeed, HBEGF was not upregulated in term trophoblasts during hypoxia, based on a semi-quantitative immunohistochemical approach previously shown to assess relative levels of HBEGF reliably in tissues [21] and cultured cells [20]. The inability of term trophoblast cells to engage the HBEGF-mediated hypoxia survival mechanism operative in the first trimester could contribute to the physiologic intolerance of term trophoblast tissues to low O₂ levels.

In vivo, stress upon the trophoblast mounts as gestation proceeds. Their success in surviving the challenges of increased demand by the growing fetus and maternal systemic changes brought about by pregnancy could determine pathologic outcomes, including preeclampsia and intrauterine growth restriction. HBEGF is a member of a family of growth factors related to EGF that activate ErbB/HER tyrosine kinase receptors [23]. HBEGF is upregulated in response to injury in the kidney, muscle, and intestine [13,24,25]. Its exogenous application protects against apoptosis, as well as ischemia or reperfusion injury [26,27], as demonstrated here for term trophoblasts exposed to hypoxia. Although HBEGF was not induced in the term trophoblast by hypoxia, its basal level of activity was required for survival during explant culture. Therefore, endogenous expression of HBEGF could be expected to moderate stress encountered by placental tissues in the course of gestation.

The trophoblast cell invasion-promoting [28] and anti-apoptotic [20] activities of HBEGF place this molecule at a point of convergence of the pathophysiological abnormalities associated with preeclampsia that include inadequate trophoblast invasion and excessive cell death [1,2]. Apoptosis occurs normally in villous trophoblasts during pregnancy [29] and is elevated in trophoblast populations of patients with preeclampsia [3,4]. We report here that a concentration of 1 nM

Fig. 1. Micrographs of chorionic villi cultured for 24 h at 20% (A) or 2% (C) O₂, for 2 h at 2% O₂ (B) and for 24 h at 2% O₂ in the medium supplemented with 1 nM HBEGF (D). Arrows indicate nuclei of trophoblast cells stained red by TUNEL. Healthy nuclei counterstained with hematoxylin appear blue. Bar = 50 μm.
HBEGF eliminated trophoblast cell death during villous explant culture at 2% O2. This concentration of HBEGF was previously found to be secreted into the medium by a first trimester trophoblast cell line exposed to 2% O2 and to protect against apoptosis [20]. These cells were also protected by 1 nM HBEGF in medium alone or medium supplemented with 1 nM recombinant HBEGF at normoxia (N/HBEGF) or hypoxia (H/HBEGF). In [B] 10 μg/ml CRM197 was added to the medium during culture at 20% O2 to antagonize HBEGF signaling. *, p < 0.05, compared to the control (0 h).

Fig. 2. The effect on trophoblast cell death of villous explant culture at 20% (normoxia) or 2% (hypoxia) O2. In (A) the TUNEL index was determined during culture in the medium alone or medium supplemented with 1 nM recombinant HBEGF at normoxia (N/HBEGF) or hypoxia (H/HBEGF). In (B) 10 μg/ml CRM197 was added to the medium during culture at 20% O2 to antagonize HBEGF signaling. *, p < 0.05, compared to the control (0 h).

conjunct with its anti-apoptotic activity [30]. The observed levels of nuclear Ki67 expression were unperturbed during in vitro explant culture for 24 h at either 2% or 20% O2. This contrasts sharply with the reported stimulation of proliferation by low O2 in first trimester human cytotrophoblast cells [32,33]. Moreover, inhibition of endogenous HBEGF signaling only transiently affected proliferation, prior to the time when apoptosis increased. We suggest that this modest reduction in proliferation represents physiological stress preceding apoptosis rather than a requirement for HBEGF in cell replication.

First trimester trophoblast cells subjected to hypoxia specifically upregulate HBEGF protein by 100-fold within 4 h through an autocrine positive feedback loop that inhibits apoptosis [20]. The human trophoblast transforms between the first trimester and later stages in its ability to survive under hypoxic conditions. During the first trimester, hypoxia increases trophoblast proliferation [32,33], but it induces cell death in trophoblast cells obtained from midterm or term placentas [16,17,34]. Therefore, we have examined the effect of O2 concentration on the expression of HBEGF in term villous explants. Term villous trophoblast cells did not upregulate HBEGF at low O2, as demonstrated by immunohistochemistry and semi-quantitative image analysis. Thus, the term trophoblast appears to lack this HBEGF-mediated hypoxia survival mechanism. After the 10th week of gestation, O2 levels rise considerably in the intervillous space and at sites of deep extravillous trophoblast invasion [35,36], greatly reducing trophoblast exposure to hypoxic conditions. Therefore, the pressure to maintain the capacity to survive at low O2 concentrations is minimal. Considering the numerous other sources of stress encountered by trophoblast cells during the final half of gestation and the ability of HBEGF to protect against cell death, the absence of HBEGF in placental tissues of women with preeclampsia could have significant pathophysiological consequences.
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References


