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Human trophoblast survival at low oxygen concentrations requires metalloproteinase-mediated shedding of heparin-binding EGF-like growth factor

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Heparin-binding EGF-like growth factor (HBEGF), which is expressed in the placenta during normal pregnancy, is downregulated in pre-eclampsia, a human pregnancy disorder associated with poor trophoblast differentiation and survival. This growth factor protects against apoptosis during stress, suggesting a role in trophoblast survival in the relatively low O_2 (~2%) environment of the first trimester conceptus. Using a well-characterized human first trimester cytotrophoblast cell line, we found that a 4-hour exposure to 2% O_2 upregulates HBEGF synthesis and secretion independently of an increase in its mRNA. Five other expressed members of the EGF family are largely unaffected. At 2% O_2, signaling via HER1 or HER4, known HBEGF receptors, is required for both HBEGF upregulation and protection against apoptosis. This positive-feedback loop is dependent on metalloproteinase-mediated cleavage and shedding of the HBEGF ectodomain. The restoration of trophoblast survival by the addition of soluble HBEGF in cultures exposed to low O_2 and metalloproteinase inhibitor suggests that the effects of HBEGF are mediated by autocrine/paracrine, rather than juxtacrine, signaling. Our results provide evidence that a post-transcriptional mechanism induced in trophoblasts by low O_2 rapidly amplifies HBEGF signaling to inhibit apoptosis. These findings have a high clinical significance, as the downregulation of HBEGF in pre-eclampsia is likely to be a contributing factor leading to the demise of trophoblasts.

KEY WORDS: Trophoblast, Placenta, Oxygen, HBEGF (HB-EGF), Metalloproteinases, Apoptosis, Human, Pregnancy

INTRODUCTION

The placenta constitutes a feto-maternal interface that is essential for survival of the developing fetus in eutherian mammals. Fetal growth is extremely rapid at the beginning of pregnancy and the placenta must enlarge to meet demand. In humans, placentation is hemochorial, and cells of the trophoblast lineage are in direct contact with the maternal blood. Oxygen supplied by maternal blood in the intervillous space crosses the trophoblast at the chorionic villi to enter the fetal circulation. Oxygen availability serves as a developmental cue to regulate trophoblast proliferation. The concentration of O_2 is relatively low (~18 mm Hg or 2%) at the human implantation site through week 10 of gestation due to occlusion of the uterine spiral arteries by extravillous trophoblasts (Rodesch et al., 1992; Jauniaux et al., 2001). Only an O_2-depleted transudate of the blood reaches the growing placenta. By the end of the first trimester, remodeling of the arteries by invading interstitial and endovascular trophoblasts completes the feto-maternal circulation, and the chorionic villi become directly exposed to maternal blood containing elevated concentrations (~60 mm Hg or 8%) of O_2. In vitro studies reveal that human cytotrophoblast proliferation rates are enhanced at low (2%) O_2 concentrations (Genbacev et al., 1996; Kilburn et al., 2000; Caniggia et al., 2000). The response of the trophoblast to low O_2 could explain the rapid placental growth achieved at the beginning of gestation.

Although many cells cannot survive when deprived of O_2, cytotrophoblast cells are resistant to hypoxia-induced apoptosis (Kilani et al., 2003). Why certain cell types (e.g. embryonic, cancer) survive at low O_2 is poorly understood (Vaupel, 2004; Schipani, 2005; Ezashi et al., 2005), although there is growing information about O_2 sensing and adaptive mechanisms (Giaccia et al., 2004). Reduced O_2 has been correlated at the molecular level with altered trophoblast expression of growth factors, integrins, glycolytic enzymes, stress-related proteins and transcription factors (Genbacev et al., 1996; Kilburn et al., 2000; Caniggia et al., 2000; Hoang et al., 2001). Autocrine signaling by cytotrophoblast cells cultured at low O_2 could inhibit apoptosis. For example, signaling downstream of the epidermal growth factor (EGF) receptor can prevent cytotrophoblast cell death during culture at very low (<10 mm Hg) O_2 (Mackova et al., 2003). However, it is not known whether activation of this receptor normally occurs or if EGF signaling is central to cytotrophoblast survival under reduced O_2 conditions.

The EGF receptor is the founding member of a receptor tyrosine kinase family (HER1-4, also known as ERBB1-4) that binds EGF and related growth factors (Holbro and Hynes, 2004). A member of the EGF family, heparin-binding EGF-like growth factor (HBEGF, also known as HB-EGF), is upregulated by hypoxia in neurons and intestinal epithelia (Tanaka et al., 1999; Jin et al., 2002; Xia et al., 2003), and functions as a mitogen and potent survival factor during stress (Pillai et al., 1998; Iwamoto and Mekada, 2000; Michalsky et al., 2001). Apoptosis induced by either transforming growth factor (TGF)-β or tumor necrosis factor (TNF)-α in human endometrial stromal cells is reduced by HBEGF (Chobotova et al., 2005). HBEGF expression is induced by sex steroids during the secretory phase of the endometrial cycle, and persists during early pregnancy

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(Das et al., 1994; Zhang et al., 1994; Yoo et al., 1997; Leach et al., 1999; Leach et al., 2001), suggesting a role in blastocyst implantation and placentation.

The accumulation of HBEGF in implantation sites could activate signaling downstream of its receptors (HER1 and HER4) to maintain cytotrophoblast survival in the low O_2 environment. During the first trimester, HBEGF is expressed by both villous and extravillous trophoblasts (Leach et al., 1999). Expression persists throughout gestation in normal pregnancies, but HBEGF is downregulated in women with the hypertensive pregnancy disorder pre-eclampsia (Leach et al., 2002b). Pre-eclampsia is associated with deficiencies in extravillous trophoblast invasion and remodeling of the spiral arteries in conjunction with elevated apoptosis (Brosens et al., 1972; DiFederico et al., 1999). HBEGF and other members of the EGF family stimulate cytotrophoblast invasiveness in vitro through interactions with HER1 and HER4 (Leach et al., 2004), suggesting that abnormally low expression of HBEGF during implantation and placentation could be a factor in the pathophysiology of pre-eclampsia.

Members of the EGF family are synthesized as type 1 transmembrane proteins that are secreted from the cell surface through metalloproteinase-mediated cleavage of their ectodomains (Holbro and Hynes, 2004). The transmembrane form of HBEGF (proHBEGF) is capable of juxtacrine signaling by binding HER1 or HER4 on adjacent cells (Iwamoto and Mekada, 2000; Harris et al., 2003). Proteolytic processing by metalloproteinases to the mature secreted form (sHBEGF) facilitates paracrine, as well as autocrine, signaling. HBEGF binding to its receptors is absolutely dependent on its interaction with heparan sulfate at the cell surface. A variety of physiological stimuli, including radiation, stress and agonists of G protein-coupled receptors, can transactivate HER1 by activating metalloproteinases responsible for the ectodomain shedding of EGF family members, particularly of HBEGF (Prenzel et al., 1999; Harris et al., 2003).

EGF family signaling could activate trophoblast survival pathways during the relatively hypoxic period of placentation in the first trimester. Therefore, we have investigated the role of this signaling network in O_2-regulated proliferation and survival of HTR-8/SVneo cells, which are derived from first trimester human cytotrophoblasts (Graham et al., 1993). Like primary cytotrophoblasts (Genbacev et al., 1996), this cell line undergoes accelerated growth when cultured in an atmosphere of 2% O_2 (Kilburn et al., 2000). Physiologically, trophoblasts do not live at 20% O_2, used in our experimental model to provide an oxygenated state, but they do experience levels near 2% during the first trimester. We hypothesized that interruption of endogenous HER signaling would be detrimental to cytotrophoblast survival and growth at 2% O_2. Our findings reveal a novel mechanism that could contribute to the survival of human cytotrophoblast cells during placentation.

MATERIALS AND METHODS

Cell culture and oxygen regulation

HTR-8/SVneo cytotrophoblast cells were plated and grown in serum-containing medium, which was replaced with serum-free medium 24 hours prior to all experiments, as previously described (Kilburn et al., 2000). The cell line was routinely phenotyped to assure cytokeratin-7 expression, hCG secretion, invasion of Matrigel™ basement membrane and HLA-G expression by invading cells. A humidified 5% CO_2/95% air incubator was used for ambient cell culture (20% O_2). Culture at low O_2 was achieved by placing cell cultures into a humidified modular incubator chamber (Billups-Rothenberg, Del Mar, CA) that was then flushed for 5 minutes with a mixture of 2% O_2, 5% CO_2 and 93% N_2 (Wilson Gases, Detroit, MI). The concentration of the gas mixture was checked using an electronic oxygen analyzer (Billups-Rothenberg) to confirm an O_2 concentration of 2%. The chamber was sealed and incubated at 37°C. The 5-minute flushing procedure was repeated after 1 hour. A cotton indicator infused with an O_2-sensitive dye (0.001% Resazurin) was placed in the incubator to ensure that a low O_2 environment was maintained throughout the incubation period. The O_2 concentration of culture medium exposed for 2 hours to this gas mixture was estimated to be ~21 mm Hg using an oxygen electrode (ISO-OXY-100, World Precision Instruments, Sarasota, FL). The electrode was calibrated using buffer solution reduced to 0% O_2 with sodium dithionite and ambient buffer (20% O_2). During flushing with the low O_2 gas mixture, O_2 levels declined to their lowest value within 2 minutes. This level was maintained in the sealed chamber for up to 24 hours.

Cell treatments

Medium was changed to add vehicle or supplements to growing cell cultures 30 minutes before exposure to ambient or 2% O_2, which initiated each experiment. The supplements included 10 μg/ml CRM197 (EMD Biosciences, San Diego, CA), 0.1 U/ml heparitinase I (from *Flavobacterium heparinum*, EC 4.2.2.8; 100704-1, Seikagaku America, East Falmouth, MA), 10 μg/ml goat anti-HBEGF (R&D Systems, Minneapolis, MN), 10 μg/ml mouse anti-HER1 (Ab-2) or HER4 (Ab-3) blocking antibodies (Lab Vision, Fremont, CA), 20 μg/ml mouse non-immune IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), 0.1-10 nM recombinant human HBEGF, EGF, TGFα, epiregulin, amphiregulin or betacellulin (R&D Systems), 2 μg/ml of the capase inhibitors, Z-DEVD-FMK, Z-IETD-FMK, Z-LEHD-FMK or Z-VAD-FMK (EMD Biosciences), 200 μg/ml of the negative control for caspase inhibitors Z-FA-FMK (EMD Biosciences), 1-20 μg/ml GM6001 {N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide} or a negative control analog, N-*t*-butoxycarbonyl-L-leucyl-L-tryptophan methylamide (EMD Biosciences).

Immunohistochemistry

Cytotrophoblast cells grown in 150 μl of medium in 96-well tissue culture plates (Becton Dickinson) were processed for immunohistochemistry, as previously described (Kilburn et al., 2000). Nuclei expressing Ki-67 were labeled with a monoclonal antibody (Ki-S5; DAKO, Carpinteria, CA). Goat polyclonal antibodies (R&D Systems) against human recombinant HBEGF (5 μg/ml), epiregulin (5 μg/ml), amphiregulin (8 μg/ml), betacellulin (5 μg/ml), EGF (8 μg/ml) and TGFα (8 μg/ml) were used at concentrations in the linear portion of each binding curve. Controls were incubated with 10 μg/ml non-immune IgG (Jackson ImmunoResearch). Cells labeled with goat primary antibodies were incubated 1 hour at 25°C with 0.1 μg/ml rabbit anti-goat IgG (Jackson ImmunoResearch). To visualize and quantify (gray level) antigen, an Envision System™ peroxidase anti-mouse/rabbit kit (DAKO) was used in conjunction with image analysis, according to our published procedure (Leach et al., 2002b). Values obtained with IgG substituted for primary antibody were subtracted from each sample.

Cell death and proliferation assays

Cytotrophoblast cells were grown and treated in 150 μl of medium in 96-well plates prior to assay. Cell death was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) using a fluorescein-based kit from Roche Applied Science (Indianapolis, IN) and counterstaining with 5 μg/ml 4′,6-diamidino-2-phenylindole, HCl (DAPI; EMD Biosciences). Fluorescent nuclei were viewed at 20× magnification using a Leica (Wetzlar, Germany) DM IRB epifluorescence microscope and representative images of both DAPI and fluorescein fluorescence were acquired with a Hamamatsu Orca digital camera (Hamamatsu City, Japan). Images were processed using Simple PCI (C-Imaging, Cranberry Township, PA) imaging software, adjusting the threshold to optimize automated counting of fluorescent nuclei. The percentage of TUNEL/DAPI-labeled nuclei (TUNEL index) was determined from triplicate fields in each well. Cells labeled with antibody against Ki-67 were counterstained with DAPI and similarly assessed for the percentage of Ki-67/DAPI-labeled nuclei as an index of cell proliferation. Externalized phosphatidylserine was detected by incubating live cells for 15 minutes at room temperature with biotin-labeled annexin V (1:20; Molecular Probes, Portland, OR) in 10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$ (pH 7.0).

DEVELOPMENT

Cells were then fixed and incubated overnight at 4°C with 5 μg/ml UltraAvidin-Texas Red (Leinco Technologies, St Louis, MO) and 5 mg/ml BSA in PBS. After counterstaining with DAPI, representative images of both DAPI and Texas Red fluorescence were acquired.

Apoptotic DNA laddering was assessed using a kit from Roche Applied Science. Cells were grown and treated in 3 ml of medium in Falcon six-well plates (Becton Dickinson). Each lane of a 2% agarose gel was loaded with 5 μg of isolated DNA, electrophoresed and stained with ethidium bromide. Gels were photographed with an EDAS290 digital camera documentation system (Kodak, New Haven, CT) using transilluminated UV light.

Cell lysis (necrosis) was assessed by measuring lactate dehydrogenase (LDH) activity released into the culture medium with a DHL^{TM} Cell Cytotoxicity Assay Kit (AnaSpec, San Jose, CA), according to the manufacturer's instructions. Cells grown in black, clear-bottom 96-well tissue culture plates (Corning, Corning, NY) were treated (n=18) as detailed in the Results in 100 μl of modified BWW medium (Irvine Scientific, Santa Ana, CA). After medium was collected for assay in separate wells, cells were washed three times with BWW medium and lysed for assay of total LDH. The fluorescent reaction product was quantified using a SpectraMax M2 multiplate spectrofluorometer (Molecular Devices, Sunnyvale, CA). To calculate the LDH release index, the value of LDH activity in the medium, multiplied by 100, was divided by the value obtained from the lysed cells.

Northern blotting and real time RT-PCR

Cytotrophoblast cells grown and treated in 2 ml of medium in six-well plates were washed three times with sterile PBS (Cambrex Bio Sciences, Verviers, Belgium) and RNA was extracted using RNeasy Mini Kits from Qiagen (Valencia, CA). Northern blotting (10 μg RNA per lane) and quantitative real time RT-PCR (1 μg RNA per reaction) were conducted as previously described (Leach et al., 2002a). Northern blots were probed with a 658-bp, [^{35}S]-labeled complementary RNA fragment of the human *HBEGF* gene prepared from a cloned cDNA, as previously described for in situ hybridization (Leach et al., 1999). The primers 5′-TGG TGC TGA AGC TCT TTC TGG-3′ (sense) and 5′-GTG GGA ATT AGT CAT GCC CAA-3′ (antisense) were used to measure *HBEGF* mRNA copy number by real time PCR. An *HBEGF* standard curve was constructed using a pGEM-7Zf(+) vector (Promega, Madison, WI) containing a human *HBEGF* insert (Accession Number M60278). TATA box binding protein mRNA, amplified using the primers 5′-CAC GAA CCA CGG CAC TGA TT-3′ (sense) and 5′-TTT TCT TGC TGC CAG TCT GGA C-3′ (antisense), was quantified in each sample to normalize *HBEGF* mRNA levels and compensate for intersample variations in the efficiency of RNA isolation and reverse transcription.

HBEGF ELISA

Cells were cultured and treated in six-well plates. Culture medium (10 ml/plate) cleared by centrifugation at 5000 ***g*** for 5 minutes was frozen at –70°C. Attached cells were extracted with 600 μl/plate of PBS containing 0.5% Tween 20 (Sigma) and protease inhibitors (1 μg/ml each of leupeptin, chymostatin and pepstatin, 25 KIU/ml aprotinin, 2 μg/ml antipain and 10 μg/ml benzamidine; all from Sigma). Extracts were centrifuged at 5000 ***g*** and the supernatants were stored frozen at –70°C. Total cellular protein concentrations were determined (Lowry et al., 1951) for all cell lysates.

Microtiter plates (Costar #3590, Corning, Corning, NY) were coated for 24 hours at 4°C with 100 μl of 0.5 μg/ml monoclonal anti-HBEGF (R&D Systems) and blocked overnight at 4°C with 1% BSA in PBS. Cell lysates were diluted 5-fold with PBS and then serially diluted for assay in PBS containing 0.1% BSA and 0.1% Tween 20. Conditioned medium was serially diluted in culture medium containing 0.1% BSA and 0.1% Tween 20. Separate standard curves were constructed for medium and lysates by preparing 0–1000 ng/ml recombinant human HBEGF (R&D Systems) in diluent containing 0.1% BSA, 0.1% Tween 20 and either culture medium or PBS, respectively. All preparations (100 μl) were incubated for 1 hour at 25°C in duplicate, antibody pre-coated, wells. After washing four times with 200 μl PBS, 100 μl of 200 ng/ml of biotin-labeled, affinity-purified, polyclonal anti-HBEGF (R&D Systems) was added to each well for 1 hour at 25°C. The wells were again washed and then incubated for 1 hour at 25°C with 100 μl of streptavidin-conjugated horseradish peroxidase (1:200; R&D Systems). The wells were washed and peroxidase activity was detected using a kit (R&D Systems) containing H_2O_2 and 3,3′,5,5′ tetramethylbenzidine. The optical density at 450 nm was determined using a programmable multiplate spectrophotometer (Power Wave Workstation, Bio-Tek Instruments, Winooski, VT). The calculated inter- and intra-assay coefficients of variation for this human HBEGF immunoassay were 3.45% and 2.26%, respectively. The detection limit of the assay was 12 pg/ml. The HBEGF concentration of each sample was calculated by interpolation from the corresponding standard curve and was expressed as picogram per microgram cellular protein.

Western blotting

Conditioned medium was collected as described for ELISA. Cell lysates were extracted in SDS sample buffer. SDS gel electrophoresis (Laemmli, 1970) was conducted under reducing conditions using 20 μg/lane of total protein. Recombinant human HBEGF (20 ng) and biotinylated protein standards (Cell Signaling Technology, Beverly, MA) were run in adjacent lanes. Proteins transferred electrophoretically to 0.45 μm nitrocellulose membranes (Fisher Scientific) were blocked for 1 hour at 25°C with 5% BSA in Tris-buffered saline containing 0.1% Tween-20. Membranes were incubated for 18 hours at 4°C with 5 μg/ml of goat anti-HBEGF in 3% BSA, and then for 1 hour at 25°C with 0.2 μg/ml peroxidase-conjugated donkey anti-goat antibody (Jackson ImmunoResearch). Labeled bands were visualized by enhanced chemiluminescence using Hyperfilm ECL (Amersham Pharmacia Biotech, Piscataway, NJ).

Statistics

Assays were conducted using replicate samples and all experiments were repeated at least three times. The program SPSS version 12.0 (SPSS, Chicago, IL) was used to determine statistical significance. For immunohistochemical quantification, the level of each growth factor at 20% and 2% O_2 was compared with a two-tailed Student's independent t-test. Comparisons were made to vehicle-treated controls for Ki-67, TUNEL and LDH data, and to 20% O_2 controls (0 minutes at 2% O_2) for RT-PCR data, all using an ANOVA with the Newman-Keuls posthoc test. TUNEL data that did not meet the assumption of equal variances among groups were log transformed before analysis. All ELISA data were analyzed by the Kruskal-Wallis non-parametric ANOVA with the Mann-Whitney posthoc test, using the Holm modification to the Bonferonni correction. All graphed data are presented as mean±s.e.m.

RESULTS

Growth factor expression at low O_2

To determine whether low O_2 alters the expression of EGF-related growth factors, six family members were examined by immunohistochemistry and were found to be expressed in cytotrophoblast cells (Fig. 1A). A semiquantitative method using image analysis provided an estimate of the effect of reduced O_2 on the expression of each growth factor. Immunostaining of HBEGF increased 3.2-fold (P<0.001) in cells cultured at 2% O_2, whereas EGF, TGFα, amphiregulin and betacellulin were unaffected by O_2 tension (Fig. 1B). Epiregulin labeling increased 1.6-fold (P=0.042), well below the effect on HBEGF.

Western blotting failed to detect HBEGF in cell lysates or conditioned medium from cytotrophoblast cells cultured for 24 hours at ambient O_2 levels, although the antibody recognized recombinant HBEGF (Fig. 2A). When O_2 was reduced to 2%, bands corresponding to pro-HBEGF (18.7 kDa) and the mature sHBEGF (9.7 kDa) appeared in cell lysates and sHBEGF was present in conditioned medium.

HBEGF, quantified by ELISA, increased dramatically in cell lysates after 4 hours of exposure to 2% O_2, from less than 0.1 pg/μg cellular protein to approximately 20 pg/μg (Fig. 2B). Secretion of HBEGF into the medium was essentially undetectable during

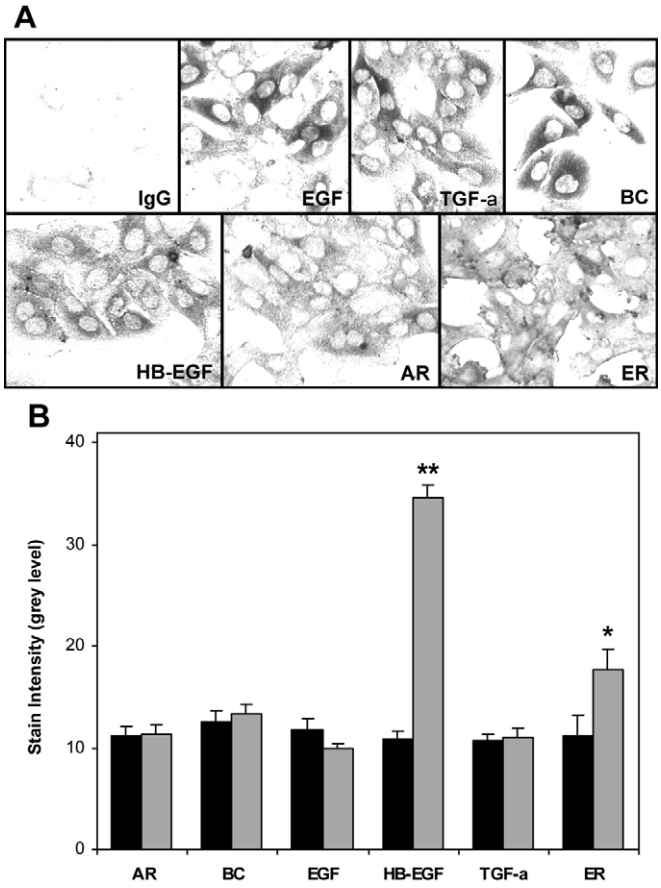

Fig. 1. Regulation of EGF family expression by O_2. (**A**) Immunohistochemical labeling of EGF family members in cytotrophoblast cells. Antibodies against EGF, TGFα (TGF-a), HBEGF, amphiregulin (AR), betacellulin (BC) and epiregulin (ER) were used, as well as a non-immune goat IgG control. (**B**) Antibody labeling was quantified by image analysis in cells cultured for 24 hours at either ambient O_2 levels (black bars) or 2% O_2 (gray bars). **P*<0.05; ***P*<0.001, compared with ambient culture.

ambient culture; however, it accumulated after 4 hours at 2% O_2 to over 500 pg/μg cellular protein and attained a concentration in the medium of approximately 1.5 nM.

Oxygen appeared to have no effect on *HBEGF* mRNA levels. Northern blotting produced a single band of the expected size (2.5 kb; Fig. 2C) with similar intensity before and after exposure to low O_2. Semi-quantitative real-time RT-PCR also revealed a constant level of *HBEGF* mRNA in cytotrophoblasts as they were switched from ambient conditions to 2% O_2 (Fig. 2D). The pool of approximately 2500 copies/cell of *HBEGF* mRNA that is maintained by trophoblasts could facilitate the upregulation of HBEGF during exposure to low O_2 through either increased synthesis or decreased degradation of the protein.

Regulation of apoptosis by HBEGF

To determine the function of HBEGF in cytotrophoblast cells exposed to low O_2, its ability to signal through its receptors, HER1 and HER4, was inhibited and the effects on cell proliferation and survival were assessed (Table 1). HTR-8/SVneo cells express all four members of the HER family (Leach et al., 2004), making them fully capable of transducing the HBEGF signal. CRM197 is a mutant diphtheria toxin that specifically antagonizes HBEGF by masking its EGF domain (Mitamura et al., 1995). HBEGF signaling was blocked with CRM197, polyclonal anti-HBEGF or a combination of function-blocking antibodies against HER1 and HER4. Disruption of HBEGF signaling did not prevent the 4-fold increase in Ki-67 expression (P<0.05) at reduced O_2. This finding indicates that HBEGF accumulation at 2% O_2 is not responsible for the increased proliferation of cytotrophoblasts.

Cytotrophoblast survival at 2% O_2, revealed by the TUNEL assay for cell death, was significantly compromised when similar treatments were used to block HBEGF signaling, or when heparitinase-mediated removal of cell surface heparan sulfate was used to deprive HBEGF of an essential cofactor for its binding to its receptor (Table 1). Interruption of HBEGF signaling had no detrimental effect on cells cultured at ambient O_2.

Cell death at low O_2 due to interference with HBEGF signaling was mediated through the apoptotic pathway, on the basis of several lines of evidence. Genomic DNA from cells treated with CRM197, anti-HBEGF or antibodies against HER1 and HER4 formed the hallmark laddering pattern of oligonucleosomal fragmentation (Fig. 3A). Pyknotic nuclei identified by DAPI labeling were TUNEL positive (Fig. 3B). Dying cells bound annexin V (Fig. 3C), indicative of the phosphatidylserine redistribution observed in apoptotic cells (Allen et al., 1997). Furthermore, this cell death could be prevented by inhibitors of the caspase cascade, but not by an inactive analog (Fig. 3D). LDH release into the medium, a sign of membrane damage during necrotic cell death, did not increase (P=0.49) when cells cultured for 8 hours at 2% O_2 were exposed to CRM197 (vehicle control, 7.57±1.11; CRM197-treated, 7.54±0.99), although a 2-hour treatment with 1 mM H_2O_2 increased (P<0.0001) LDH release (33.0±1.55). These data provide compelling molecular and biochemical evidence that HBEGF signaling prevents activation of the apoptotic pathway in cytotrophoblasts cultured at 2% O_2.

Regulation of HBEGF

Decreased survival at 2% O_2 under conditions that inhibited HBEGF signaling was accompanied by a failure to upregulate cellular and secreted HBEGF (Table 1), suggesting that HBEGF is self regulating. CRM197 or anti-HBEGF treatment abolished HBEGF accumulation in cytotrophoblast cells cultured at 2% O_2. Treatment with blocking antibodies against both HER1 and HER 4 inhibited the rise in cellular and secreted HBEGF. Only a partial reduction of HBEGF upregulation was achieved by inhibiting either HER1 or HER4 alone. However, the residual HBEGF secretion was sufficient to maintain cell survival, as indicated by low TUNEL. Therefore, signaling downstream of either HER1 or HER4 induces HBEGF accumulation and maintains cytotrophoblast survival at 2% O_2.

The role of HBEGF secretion

Transmembrane proHBEGF can directly bind its receptors on adjacent cells (Iwamoto and Mekada, 2000), or it can be processed by metalloproteinases to sHBEGF (Prenzel et al., 1999). In the presence of the metalloproteinase inhibitor GM6001, cytotrophoblast cells cultured at 2% O_2 failed to upregulate cellular or secreted HBEGF (Fig. 4A). GM6001 inhibition was dose-dependent, with maximal effect at 10 μg/ml. HBEGF upregulation was not affected by an inactive structural analog that served as a negative control (10 μg/ml; data not shown), nor did the analog affect the rate of apoptosis at 2% O_2 (Fig. 4B). However, 10 μg/ml GM6001 reduced cytotrophoblast cell survival at 2% O_2 (Fig. 4B), but had little effect on the rise in nuclear Ki-67 expression (data not

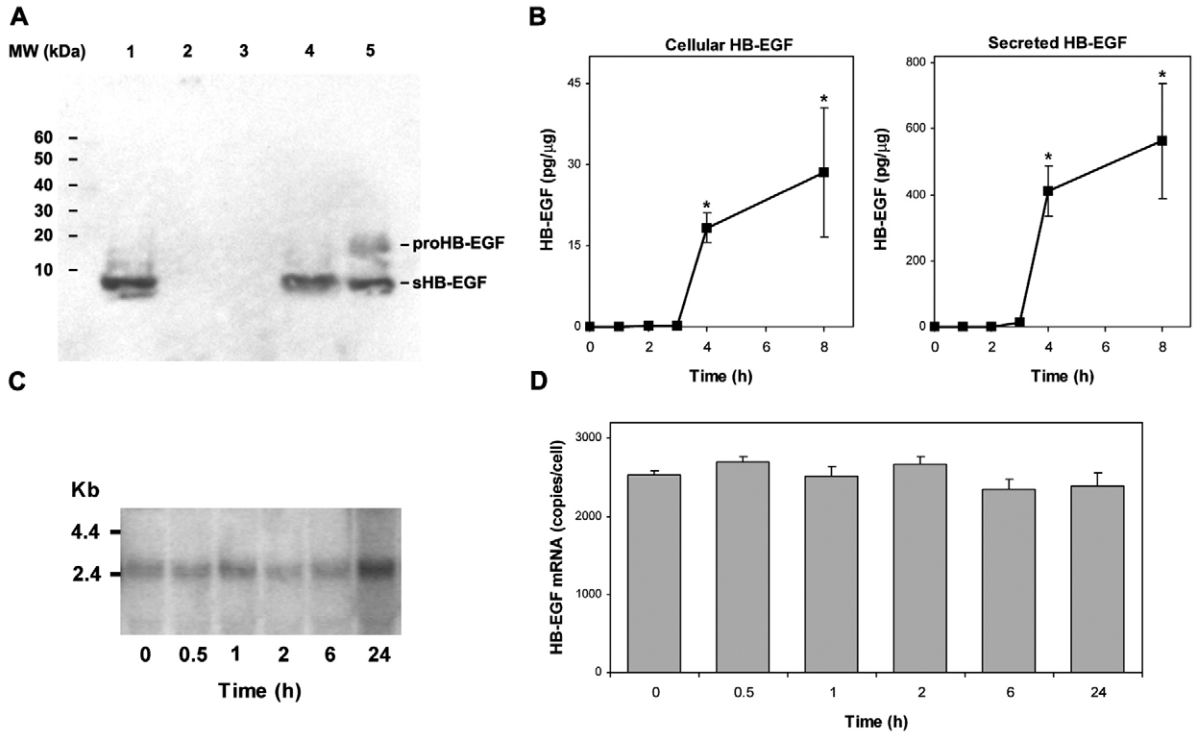

Fig. 2. Regulation of HBEGF accumulation by O_2. (**A**) Western blot of HBEGF. Recombinant human HBEGF (20 ng in lane 1) contains the same sequence as sHBEGF. HBEGF was undetectable in conditioned medium (lane 2) or in cytotrophoblast cell lysate (lane 3) during culture at ambient O_2 levels. After 24 hours at 2% O_2, a 9.7 kDa sHBEGF band was detected in the medium (lane 4), and an 18.7 kDa pro-HBEGF band appeared in cell lysates in addition to the sHBEGF band (lane 5). (**B**) Cellular and secreted pools of HBEGF were quantified by ELISA in cell lysates and conditioned medium after shifting cells to 2% O_2. *P<0.05, compared with control (0 hours). (**C**) Northern blot of the 2.5 kb *HBEGF* transcript in cytotrophoblast RNA 0-24 hours after shifting cells to 2% O_2. (**D**) Real time RT-PCR was used to quantify *HBEGF* mRNA 0-24 hours after shifting cells to 2% O_2. ANOVA indicated no significant differences.

shown). Neither compound altered the apoptosis of fully oxygenated cells. The inhibitory activities of GM6001 support a mechanism, independent of juxtacrine HBEGF signaling, that is initiated by shedding from a small pool of proHBEGF maintained on the surface of trophoblasts.

By inhibiting metalloproteinase activity, we hypothesized that cytotrophoblasts exposed to 2% O_2 are deprived of sHBEGF essential for the activation of HER1 or HER4. Indeed, the ability of GM6001 to hinder cell survival under conditions of low O_2 was overcome by providing recombinant human HBEGF in the culture medium. Cell death was ameliorated by exogenous HBEGF in a dose-dependent manner (Fig. 4D), with maximal cell survival at 1 nM HBEGF. Other members of the EGF family expressed by cytotrophoblasts, including epiregulin, failed to decrease apoptosis under these conditions when added at a concentration of 10 nM (Fig. 4D), demonstrating the specificity of HBEGF as a trophoblast survival factor.

DISCUSSION

Our findings suggest that HBEGF autocrine signaling permits human trophoblast cells to survive at the relatively low O_2 levels present in the first trimester placenta. Of the six EGF family members expressed in the HTR-8/SVneo cytotrophoblast cell line, HBEGF, rose dramatically after lowering the O_2. After 4 hours of

Table 1. Influence of low O_2 and HBEGF signaling on cytotrophoblast survival, proliferation and HBEGF production

Treatment	Concentration	Proliferation (Ki67 index)		Cell death (TUNEL index)		Cellular HBEGF (normalized)	Secreted HBEGF (normalized)
		Ambient O_2	2% O_2	Ambient O_2	2% O_2	2% O_2	2% O_2
Vehicle		11.4±1.0	41.8±2.5	4.75±0.55	4.50±0.23	100±25	100±26
CRM197	10 μg/ml	10.2±0.63	37.6±2.8	3.94±0.43	9.86±0.41*	0.943±0.34*	ND
Anti-HBEGF	10 μg/ml	9.52±0.82	34.1±2.79	3.93±0.69	11.5±0.56*	1.37±0.70*	ND
Heparitinase	0.1 U/ml			3.66±0.38	11.5±0.63*		
Anti-HER1 and HER4	10 μg/ml each	10.1±0.52	37.3±2.34	4.85±0.33	10.1±0.46*	5.51±2.5*	4.66±4.54*
Anti-HER1	10 μg/ml				4.14±0.49	57.0±33	27.7±18.7*
Anti-HER4	10 μg/ml				4.34±0.81	51.6±20	25.2±17.3*
Non-Immune IgG	20 μg/ml			5.10±0.57	5.24±0.40		

Cytotrophoblast cells were cultured at the indicated O_2 levels for either 4 (HBEGF measurement) or 8 (cell death and proliferation) hours. All treatments were initiated 30 minutes before the start of culture and continued for the remainder of the culture period. Cells and medium were collected and assayed for Ki67 expression, TUNEL and HBEGF, as described in the Materials and methods section. Mean±s.e.m. is shown for all measurements. HBEGF concentrations, determined by ELISA, were normalized as a percentage of the vehicle treatment.

*P<0.05, compared with vehicle at the same O_2 level.

ND, not done because the presence of CRM197 or anti-HBEGF in medium interferes with the HBEGF ELISA.

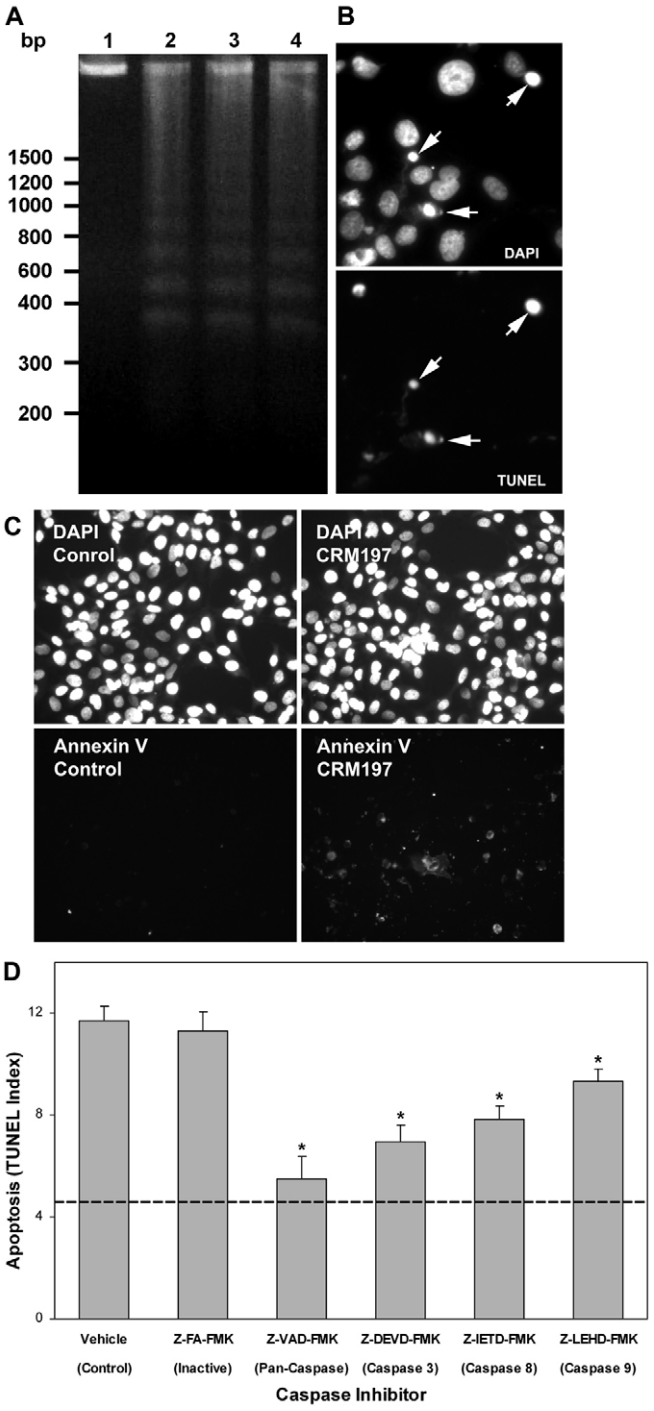

Fig. 3. Contribution of the apoptotic pathway to cytotrophoblast cell death. All cells were cultured for 8 hours at 2% O_2 in medium supplemented as described below and in the Materials and methods. (**A**) Internucleosomal cleavage of DNA was assessed by agarose gel electrophoresis and ethidium bromide staining of genomic DNA. Cells were cultured in medium containing vehicle (lane 1), CRM197 (lane 2), anti-HBEGF (lane 3), or antibodies against both HER1 and HER4 (lane 4). (**B**) Cells exposed to CRM197 were dually stained using DAPI and the TUNEL method. Pyknotic nuclei visualized by fluorescence microscopy in the upper panel were also positive for TUNEL (arrows). (**C**) Annexin V binding to phosphatidylserine exposed on the surface of apoptotic cells is nearly absent in control cells (lower left panel) compared with cells treated with CRM197 (lower right panel). The upper panels show the same fields labeled with DAPI to indicate the relative cell densities. (**D**) Cell death was quantified by TUNEL assay after culture in medium containing CRM197. The indicated caspase inhibitors (2 μg/ml) or an inactive analog (200 μg/ml) were also added to the medium. Specificity of each compound is shown in parentheses. The dashed line indicates the level of TUNEL in cells cultured without CRM197. **P*<0.05, compared with vehicle control.

culture at 2% O_2, cellular levels of HBEGF increased abruptly by over two orders of magnitude. In addition to HBEGF, only epiregulin registered a significant, albeit moderate, increase according to immunohistochemical staining (Fig. 1B). Like HBEGF, epiregulin binds both HER1 and HER4, but unlike HBEGF, it does not require heparan sulfate for binding (Holbro and Hynes, 2004). Amphiregulin is the only other EGF family member examined in this study that binds heparan sulfate. Our data indicate that the observed increase in epiregulin at 2% O_2 could not substitute functionally for HBEGF. Neither CRM197 nor anti-HBEGF antibody should block epiregulin binding, yet both inhibited the endogenous signaling required for HBEGF upregulation and cell survival. The specificity of HBEGF was further demonstrated by its ability to restore the survival of cells blocked in HBEGF secretion, while epiregulin and other EGF family members were completely ineffective. If the HTR-8/SVneo cell line is representative of primary cytotrophoblasts with respect to its responses to O_2, our findings establish HBEGF as a crucial survival factor during the first trimester in the developing human placenta. This role would be consistent with HBEGF function in other organs and developing systems where cells are exposed to relatively low O_2 levels (Horikawa et al., 1999; Tanaka et al., 1999; Jin et al., 2002; Xia et al., 2003; Schipani, 2005; Ezashi et al., 2005).

HBEGF expression in the human placenta could influence multiple physiological processes that regulate placentation to ensure a normal pregnancy. HTR-8/SVneo cell proliferation increases after lowering O_2 to 2%, as assessed by the analysis of cell number and the expression of Ki-67 (Kilburn et al., 2000). This phenomenon can also be demonstrated with freshly isolated cytotrophoblasts and villous explants (Genbacev et al., 1996; Caniggia et al., 2000). Although HBEGF was potent in preventing hypoxic injury, it appeared to have no mitogenic role and, therefore, does not mediate the hypoxic simulation of trophoblast proliferation. Supplementation with HBEGF, EGF or TGFα has little effect on proliferation rates, but is highly effective at converting these cells to an invasive phenotype (Leach et al., 2004). Therefore, HBEGF regulates trophoblast survival and invasion, two crucial functions that are compromised in pregnancies complicated by pre-eclampsia (Brosens et al., 1972; DiFederico et al., 1999). These findings are noteworthy in the light of our previous report that HBEGF is significantly downregulated in placentas of pre-eclamptic women (Leach et al., 2002b).

In the absence of HBEGF signaling, elevated cytotrophoblast cell death was mediated through the apoptotic pathway, based on observations of pyknotic nuclei, internucleosomal DNA cleavage, externalized phosphatidylserine and dependence on the caspase cascade. Z-VAD-FMK, an inhibitor of all caspases, completely blocked apoptosis, whereas the inactive analog Z-FA-FMK failed to reduce apoptosis. Inhibition of the effector caspase, caspase 3, was

more effective than inhibition of either initiator caspase, leaving it uncertain whether receptor-mediated or endogenous apoptotic signaling is involved. No evidence was found for necrosis, which would have resulted in the release of LDH into the culture medium. EGF, which activates HER1 (Holbro and Hynes, 2004), inhibits hypoxia-induced apoptosis in cytotrophoblast cells (Mackova et al., 2003), yet activators of other receptor tyrosine kinases, including basic fibroblast growth factor, insulin-like growth factor 1, platelet-derived growth factor AA, vascular endothelial growth factor (VEGF) and placental growth factor, are all ineffective (Smith et al., 2002). Trophoblast survival is mediated downstream of HER1 by the p42/p44 and JNK mitogen-activated protein kinase pathways, sphingosine kinase 1 and phosphatidylinositol 3-kinase (Mackova et al., 2003; Johnstone et al., 2005). Presumably, HBEGF operates similarly, although HER4 could recruit additional pathways. It is not known whether the same downstream pathways are responsible for the upregulation of HBEGF by low O_2.

We have examined the hypothesis that reduced O_2 initiates metalloproteinase activation and the liberation of sHBEGF to transactivate HER1 and HER4, and that downstream HER signaling increases HBEGF levels in a positive-feedback loop (Fig. 5). The inhibitory activities of GM6001 support a mechanism that is independent of juxtacrine HBEGF signaling and suggest that a small pool of transmembrane proHBEGF provides the substrate for metalloproteinases once O_2 is reduced. HER transactivation by stimuli that activate metalloproteinases to induce HBEGF shedding is a well-established cellular mechanism (Prenzel et al., 1999; Harris et al., 2003). We should note that reactive oxygen species, which are generated by hypoxia (Zuo and Clanton, 2005), are among the stimuli that can induce HBEGF shedding (Frank et al., 2003; Kim et al., 2005). HER1 and HER4 were responsible for transducing HBEGF signaling, based on inhibition studies using antibodies directed against their extracellular domains. We have used these antibodies previously to inhibit integrin switching induced by EGF family members in HTR-8/SVneo cells (Leach et al., 2004). Although inhibiting HBEGF binding to either HER1 or HER4 alone diminished the upregulation of HBEGF at 2% O_2, there remained sufficient HBEGF secreted (~25% of control) to avert apoptosis. Interestingly, this was approximately the concentration of exogenous HBEGF required to rescue cytotrophoblasts treated with GM6001 during culture at 2% O_2. These data establish a post-transcriptional mechanism for cytotrophoblast survival at low O_2, based on the

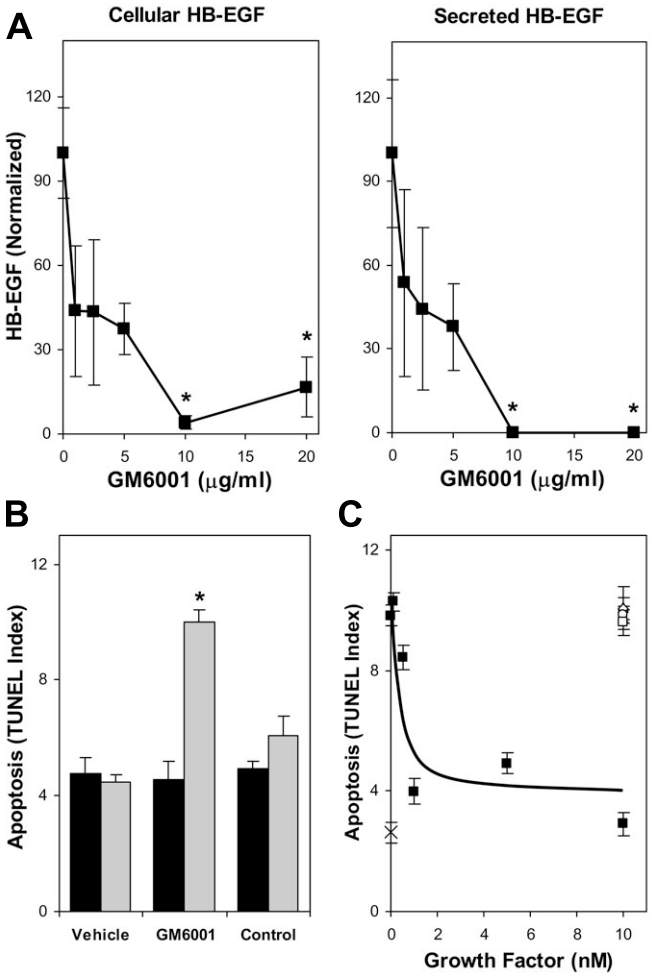

Fig. 4. Role of HBEGF shedding at 2% O_2. (**A**) Cellular and secreted HBEGF concentrations were measured by ELISA after culture for 8 hours at 2% O_2 in medium containing the indicated concentrations of GM6001. HBEGF values were normalized to vehicle, as in Table 1. **P*<0.05 compared with vehicle. (**B**) Cytotrophoblast cells were cultured for 8 hours at ambient O_2 levels (black bars) or 2% O_2 (gray bars) in medium supplemented with vehicle, 10 μg/ml GM6001 or 10 μg/ml of an inactive structural analog (Control) and TUNEL was determined. **P*<0.05 compared with vehicle treatment. (**C**) Cells were cultured for 8 hours at 2% O_2 with 0 μg/ml (×) or 10 μg/ml (all other symbols) GM6001 and apoptosis was determined by TUNEL. The indicated concentrations of recombinant human HBEGF (black squares) were added to GM6001-treated cells. Co-treatment was also conducted with 10 nM of other recombinant EGF family growth factors, including epiregulin (open circle), betacellulin (open diamond), amphiregulin (open triangle), EGF (open square) or TGFα (solid triangle). The 0 μg/ml GM6001 control was different (*P*<0.05) from all other treatments except those containing 10 μg/ml GM6001 plus 1–10 nM HBEGF.

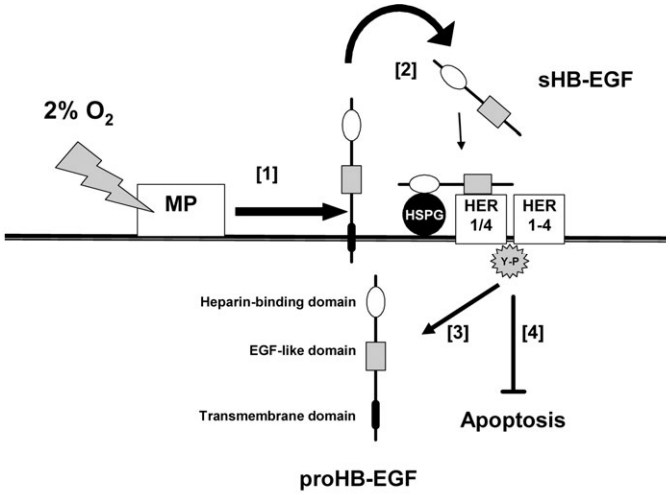

Fig. 5. Proposed mechanism for cytotrophoblast survival at 2% O_2. Activated metalloproteinases (MP) at the cell surface cleave the extracellular domain of proHBEGF (1). The released sHBEGF binds to HER1 or HER4 through its EGF-like domain and to heparan sulfate proteoglycans (HSPG) through its heparin-binding domain (2), and this is followed by receptor homo- or heterodimerization with other members of the HER family. Subsequent transphosphorylation of HER cytoplasmic domains at key tyrosine residues (Y-P) initiates downstream signaling that increases proHBEGF accumulation (3) and inhibits apoptosis (4). This positive feedback loop upregulates HBEGF secretion to achieve extracellular HBEGF levels sufficient to maintain cell survival at 2% O_2.

transactivation of HER1 or HER4 through sHBEGF shedding, leading to both HBEGF amplification and inhibition of apoptosis (Fig. 5). This response contrasts with reports of brain neurons and intestinal epithelial cells, in which HBEGF is upregulated by hypoxia at the mRNA level (Tanaka et al., 1999; Xia et al., 2003).

Oxygen, which is low in the first trimester, regulates trophoblast phenotype during normal development (Jauniaux et al., 2001). HBEGF is upregulated by cytotrophoblasts 4 hours into O_2 deprivation, providing an early, crucial response to hypoxia. Human trophoblasts also upregulate VEGF, soluble FLT1, TGFβ3, TNFα, IL1α and IL1β (Benyo et al., 1997; Caniggia et al., 2000; Ahmed et al., 2000; Nagamatsu et al., 2004; Nishi et al., 2004; Li et al., 2005). The levels of these proteins increase much later (24-72 hours at 2% O_2) and might contribute to trophoblast survival or stimulate proliferation. Hypoxia inducible factor (HIF)-1α is a transcription factor produced by human cytotrophoblasts within 4 hours of exposure to reduced O_2 (Caniggia et al., 2000; Hayashi et al., 2005). Although the timing of HIF1α expression would be consistent with a role upstream of HBEGF accumulation, it would have to operate indirectly, because HBEGF is regulated by O_2 at the post-transcriptional level. Within a similar timeframe, tissue inhibitors of metalloproteinases (TIMP) 1 and 2 are downregulated, suggesting the possibility of increased matrix metalloproteinase (MMP) activity (Canning et al., 2001) that could directly mediate HBEGF shedding, as outlined in Fig. 5. HBEGF shedding can be regulated by a variety of metalloproteinases in the MMP and ADAM (a disintegrin and metalloproteinase) families (Yu et al., 2002; Razandi et al., 2003; Wu et al., 2004; Higashiyama and Nanba, 2005).

Global gene expression by placental tissues at low O_2 resembles that of placentas from women with pre-eclampsia (Soleymanlou et al., 2005). Placental expression of HIF1α and TGFβ3, proteins associated with a less-differentiated trophoblast phenotype, persists late into gestation in pre-eclampsia (Caniggia et al., 2000). These findings suggest that the aberrant regulation of trophoblast differentiation by oxygen in pre-eclampsia impedes remodeling of the spiral arteries and diminishes perfusion of the chorionic villi. Whether the pathology is the result of hypoxia or other factors that produce a similar outcome is not known. Oxidative stress caused by reoxygenation after ischemia has also been suggested as an instigating factor in pre-eclampsia (Roberts and Hubel, 1999; Hung et al., 2002). The reduced expression of HBEGF observed in pre-eclampsia (Leach et al., 2002b) could reflect the disruption of oxygen regulation or a failure of the trophoblast to respond normally to environmental cues. The present study indicates that, in the absence of HBEGF, trophoblast cells are highly vulnerable to stresses that jeopardize their survival.

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