Apoptosis in human cultured trophoblasts is enhanced by hypoxia and diminished by epidermal growth factor

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Levy, Roni, Steven D. Smith, Kala Chandler, Yoel Sadovsky, and D. Michael Nelson. Apoptosis in human cultured trophoblasts is enhanced by hypoxia and diminished by epidermal growth factor. Am J Physiol Cell Physiol 278: C982–C988, 2000.—Preeclampsia and fetal growth restriction are associated with placental hypoperfusion and villous hypoxia. The villous response to this environment includes diminished trophoblast differentiation and enhanced apoptosis. We tested the hypothesis that hypoxia induces apoptosis in cultured trophoblasts, and that epidermal growth factor (EGF), an enhancer of trophoblast differentiation, diminishes hypoxia-induced apoptosis. Trophoblasts isolated from placentas of term uncomplicated human pregnancies were cultured up to 72 h in standard (PO2 = 120 mmHg) or hypoxic (PO2 < 15 mmHg) conditions. Exposure to hypoxia for 24 h markedly enhanced trophoblast apoptosis as determined by DNA laddering, internucleosomal in situ DNA fragmentation, and histomorphology, as well as by the reversibility of the apoptotic process with a caspase inhibitor. Apoptosis was accompanied by increased expression of p53 and Bax and decreased expression of Bcl-2. Addition of EGF to cultured trophoblasts or exposure of more differentiated trophoblasts to hypoxia significantly lowered the level of apoptosis. We conclude that hypoxia enhances apoptosis in cultured trophoblasts by a mechanism that involves an increase in p53 and Bax expression. EGF and enhancement of cell differentiation protect against hypoxic-induced apoptosis.

DNA fragmentation; p53; Bcl-2 proteins; caspase inhibitor

Fetal growth and development depend on intact placental function. The human placenta is composed of mononuclear cells, cytotrophoblasts, which coalesce to become a multinucleated syncytiotrophoblast. Maintenance of trophoblast structure and differentiated function is essential for the provision of adequate gas, nutrient, and waste exchange between the fetus and the mother. Placental trophoblasts may be subject to diverse insults during normal human pregnancy. Clinical conditions such as preeclampsia, anemia, smoking, and living in a high altitude can lead to placental underperfusion and villous hypoxia, characterized by diminished syncytial differentiation, syncytial knots, and prominent cytotrophoblasts (4). Although others and we have shown that hypoxia hinders differentiation of term trophoblasts in vitro (1, 20), the cellular and molecular mechanisms underlying trophoblast hypoxic injury are presently unknown.

Apoptosis is a component of normal development and differentiation in most tissues. This type of cell death may be enhanced by deleterious stimuli such as hypoxia (19), distorting the balance of cellular proliferation, differentiation, and death, thereby impairing placental function. Indeed, a higher degree of apoptosis is found in placentas from pregnancies complicated by fetal growth restriction (FGR) (27). Similarly, apoptosis is more prevalent in cytrophoblasts from pregnancies complicated by preeclampsia, compared with similar specimens obtained from uncomplicated pregnancies (6).

Apoptosis can be prevented by several growth factors such as nerve growth factor (31), insulin-like growth factor (15), and epidermal growth factor (EGF) (23, 30). EGF is present in high levels in the maternal and fetal circulation. EGF promotes differentiation (18) and prevents tumor necrosis factor-α (TNF-α)-induced apoptosis in cultured term trophoblasts (9). There are at least two signaling pathways known to trigger apoptosis. The first is mediated by interactions of membrane receptors and ligands, such as Fas ligand or TNF-α. The second pathway is triggered by exogenous stimuli such as hypoxia, radiation, and chemotherapeutic drugs, where the death signal is transmitted through the mitochondria. This pathway involves altered expression of p53 and members of the Bcl-2 family. Several studies suggest a role for p53 in the hypoxia-mediated apoptosis in different tissues (16, 28). In the placenta, p53 and the proapoptotic protein Bax are expressed primarily in undifferentiated cytotrophoblasts in placental villi and in cytrophoblasts of gestational trophoblastic disease, a neoplastic condition with prominent apoptosis (24). In contrast, the antiapoptotic protein Bcl-2 is expressed at higher levels in the differentiated syncytiotrophoblast (26). We used an in vitro system to examine the effect of hypoxia on apoptosis in term trophoblasts. We hypothesized that hypoxia enhances apoptosis in term human trophoblast in vitro and speculated that EGF protects against hypoxia-induced apoptosis.

MATERIALS AND METHODS

Cell isolation and culture. Placentas were obtained immediately after a term singleton delivery from women with...
uncomplicated pregnancies. Villous cytotrophoblasts were isolated by the method described by Kliman et al. (12), with modifications (20). Isolated cytotrophoblasts were plated in medium 199 (tissue culture facility, Washington University, St. Louis, MO) containing 20 mM HEPES (Sigma, St. Louis, MO) and 2 mM L-glutamine (Sigma) supplemented with 10% fetal bovine serum in 5% CO₂, 95% air at 37°C. Nonadherent cells were removed after 4 h by washing three times with PBS, and the adherent cells were transferred to either an anaerobic chamber (Forma Scientific, Marietta, OH) or maintained in standard conditions. The anaerobic chamber provided a hypoxic atmosphere, defined as 1–2% oxygen (5% CO₂, 10% H₂, and 85% N₂) that yields a PO₂ of <15 mmHg. Standard conditions were defined as 5% CO₂ and 95% air (i.e., 20% oxygen). Cells were cultured up to 72 h and were exposed to hypoxic conditions of different time intervals during the culture. In several experiments, cells were exposed to 40 µM of the broad spectrum caspase inhibitor Z-Val-Ala-Asp (Ome)-fluoromethylketone (ZVAD-fmk; Enzyme Systems Products, Livermore, CA) for 40 min before culture in hypoxia or standard conditions or to recombinant EGF (100 ng/ml; Upstate Biotechnology, Lake Placid, NY) that was added to the media either before or during exposure to hypoxic conditions. For selected experiments, the chamber’s gas mixture was adjusted to 5% oxygen (PO₂ = 60 mmHg) or 10% oxygen (PO₂ = 75 mmHg) by reducing the nitrogen concentration.

Terminal deoxynucleotidyl transferase method. Cells were washed in PBS and scraped from dishes into lysis buffer solution. The DNA was isolated and its concentration was determined by absorbance at 260 nm. Internucleosomal fragmentation of DNA was determined by the terminal deoxynucleotidyl transferase (TdT) method according to the manufacturer’s instructions (Boehringer Mannheim, Indianapolis, IN). Briefly, 0.5–1.0 µg of DNA was end labeled with α-32P deoxyadenosine triphosphate with the enzyme TdT. DNA samples were electrophoresed through a 2% agarose gel, dried, and exposed to film. For quantification of labeled DNA, each lane was cut, placed in scintillation solution (ScintiVerse BioHP; Fisher Labs, Springfield, NJ), and quantified using a beta counter.

TdT-mediated dUTP nick end labeling staining. Cells in culture dishes were dried by briefly warming over a flame and were fixed in 4% Formalin for 10 min. Endogenous peroxidase was quenched with 3% H₂O₂, and cells were washed with PBS. The ApopTag kit (Oncor, Gaithersburg, MD) was used for TdT-mediated dUTP nick end labeling (TUNEL) staining according to the manufacturer’s instructions. Briefly, cells were incubated in an equilibration buffer for 10 min and then in a 37°C humidified chamber for 1 h with TdT and dUTP digoxigenin. The reaction was stopped, and cells were washed and incubated with anti-digoxigenin peroxidase solution, colorized with amino ethylcarbazole (Vector Laboratories, Burlingame, CA), and counterstained with hematoxylin. Negative control cells were treated identically without addition to the TdT enzyme. The cells were mounted under coverslips with aqueous mounting medium (Gel Mount; Biomed, Foster City, CA) and examined by bright field microscopy. The number of TUNEL stained and unstained nuclei were counted by two examiners who were blinded to the culture conditions in five randomly selected microscopic fields at ×400 magnification providing a minimum of 200 cells. Apoptotic index was defined as the percentage of cells that were stained by the TUNEL method divided by the total number of cells counted.

Morphology. Trophoblasts stained by hematoxylin and eosin were assessed for apoptotic features using a bright field microscope (Olympus BH2). Cells were identified as apoptotic if they had condensed or fragmented nuclei, a low cytoplasm to nucleus ratio indicative of cell shrinkage, or surface membrane blebbing, all features of apoptotic cells. Apoptotic cells were counted in five randomly selected fields and expressed as a percentage of cells with apoptotic morphology divided by the total number of cells counted.

Western blot. Cells were collected in a lysis buffer that contained 10% SDS and sonicated. Samples of 40 µg/lane of protein were subjected to electrophoresis at 60 V for 2 h in 4°C using polyacrylamide gel slabs (10% for p53 and 12% for the other proteins), then transferred to polyvinylidene difluoride membranes overnight. Membranes were blocked with 5% skim milk for 1 h, incubated with monoclonal antibodies to Bax, Bak, BclX, p53 (all from Santa Cruz), and Bcl-2 (Pharmingen, San Diego, CA), washed twice for 10 min with Tris-buffered saline (TBS; 0.75% Tween 20), and then incubated for 1 h with the corresponding horseradish peroxidase-linked secondary antibodies (Santa Cruz). The membranes were then washed twice for 10 min with TBS and processed for chemiluminescence using an enhanced chemiluminescence kit (Amersham, Pharmacia, and BioTech, Piscataway, NJ). Membranes were blocked with 5% nonfat milk to firm the bands, exposed to film for 1–10 min, and the resulting bands were analyzed with a densitometer (Molecular Dynamics, Sunnyvale, CA). For quantitative estimation of Bax and Bcl-2 concentrations, the density of these proteins extracted from cultured trophoblasts to the density of serial dilutions of purified peptides at known concentrations, linked to glutathione S-transferase (Santa Cruz).

Medium human chorionic gonadotropin immunoassay. Human chorionic gonadotropin (hCG) concentration in cell culture media was determined by an ELISA (Abbott Laboratories, Abbott Park, IL) using a specific monoclonal antibody directed against the β-subunit of hCG, and normalized to DNA.

Statistical analysis. Each measurement was performed in duplicate for each condition. The number of placentas used is denoted in parentheses. Data are presented as mean ± SD. Comparisons were made using Student’s t-test, and a P < 0.05 was considered significant.

RESULTS

We initially determined the effect of varying times of hypoxia on apoptosis in cultured term human trophoblasts (n = 2). With the use of the TdT method, we found that hypoxia for ≥24 h caused a marked increase in DNA fragmentation, compared with cells exposed to standard conditions (Fig. 1A). Longer exposures to hypoxia up to 72 h did not further enhance the level of DNA fragmentation (Fig. 1B). We therefore used cultures up to 24 h in hypoxia to compare with standard conditions and completed a detailed quantification of the apoptosis that occurred in the first 24 h in culture. With the use of different term placentas (n = 7), we found a 2.8 ± 0.4-fold increase in DNA fragmentation, as assessed by the TdT method, compared with control (P < 0.001). Complementing the TdT method, the TUNEL stain revealed a similar effect (Fig. 1C). The apoptotic index of cells exposed to 24 h of hypoxia, assessed by the TUNEL method, was 32.9 ± 9.7%, compared with 5.8 ± 5.4% in the control cells (P < 0.001; Figs. 1C and 2A and B). Furthermore, hematoxylin-eosin staining confirmed the higher level of apoptosis in cells exposed to hypoxia (Fig. 2, E and F). Preincubation of the cells with 40 µM of ZVAD-fmk, a broad spectrum caspase inhibitor, markedly decreased...
the level of apoptosis to an apoptotic index of 18.8 ± 5.3% (P < 0.05), but did not abolish the enhanced apoptosis induced by 24 h of hypoxia (Fig. 2C). Taken together, these results indicate that hypoxia induces apoptosis in cultured term human trophoblasts, with a maximal effect in the first 24 h in culture.

Trophoblasts in vivo may be exposed to an oxygen level lower than the 20% used as standard conditions by others and by us (28). Therefore, we used the TUNEL method to compare the level of apoptosis in cultures exposed to standard conditions, with cultures exposed to 5 or 10% ambient oxygen (n = 3). We found that cells exposed to either 5 or 10% O₂ exhibited an apoptosis index that was indistinguishable from the apoptotic index of cells exposed to 20% O₂. We therefore continued to use cells exposed to 20% oxygen as our control.

To control for the possibility that detachment of apoptotic trophoblasts into the medium during culture might affect our analysis, we compared the amount of DNA and quantitated the apoptosis in floating and adherent cells in the same culture. With the use of DNA quantification, we determined that <5% of the cells were detached. Moreover, using the TdT as well as TUNEL assays, we found a similar degree of apoptosis in the floating cells that detached from the plates during either standard or hypoxic conditions in culture (data not shown). We concluded that the contribution of the floating cells was low enough to exclude them from our analysis.

The p53, as well as proteins from the Bcl-2 family, have been implicated in the mechanism of hypoxia-induced cell death in a variety of tissues (13, 28). We first examined the expression of p53 in trophoblasts cultured in standard or hypoxic conditions. We found a 2.9 ± 0.7-fold increase (P < 0.01) in p53 expression in trophoblasts cultured for 24 h in hypoxia, compared with control (Fig. 3). We additionally studied the effect of hypoxia on the expression of four members of the Bcl-2 family of proteins that were identified in preliminary studies to be expressed in placental villi (22) and that are involved with a key life-death rheostat for cells (13). We found that the expression of the proapoptotic protein Bax was 1.4 ± 0.4-fold higher in cells exposed to 24 h of hypoxia, compared with control (P < 0.05). In contrast, the expression of the antiapoptotic protein Bcl-2, in the same cells, diminished to 0.7 ± 0.2-fold compared with control (P < 0.01, Fig. 4). The expression of Bak and Bcl-XL was unchanged when exposed to hypoxia in culture. Exposure of trophoblasts to hypoxia at a later time interval, when cells were more differentiated (confirmed by histology and higher hCG production, data not shown), did not further alter Bax or Bcl-2 expression. To explore the significance of the hypoxia-induced alteration in the Bax/Bcl-2 ratio (2-fold), we estimated the relative expression of Bax to Bcl-2 in trophoblasts cultured in standard conditions using peptides of known concentrations. We found that the concentration of Bax was ~15 times higher than that of the Bcl-2 protein (n = 2, data not shown), which
suggests that Bax plays a prominent role in trophoblast apoptosis.

EGF promotes trophoblastic differentiation (18) and inhibits cytokine-induced apoptosis (9). We therefore tested the effect of EGF on hypoxia-induced apoptosis in trophoblasts. We assessed hCG production and confirmed that EGF enhanced trophoblast differentiation in our system as reflected by increased hCG production (data not shown). Cells exposed to hypoxia for 24 h, in the presence of EGF, exhibited a 0.6 ± 0.2-fold lower level of apoptosis, compared with cells cultured in hypoxia without EGF (P < 0.05), as determined by the TdT method (Fig. 5). Furthermore, the apoptotic index of cells exposed to hypoxia in the presence of EGF was 15.8 ± 5.5% as assessed by TUNEL staining, compared
with 35.8 ± 8.9% in cells exposed to hypoxia in the absence of EGF (P < 0.001, Fig. 2D). A similar effect, albeit to a lesser degree, was observed in cells cultured in standard conditions in the presence or absence of EGF. In contrast, exposure to transforming growth factor-β, which does not promote differentiation, did not decrease the degree of apoptosis (data not shown). Our data suggest that less differentiated trophoblasts are more susceptible to hypoxia-induced apoptosis, compared with differentiated cells. To further examine this possibility, we compared the effect of trophoblast differentiation on the susceptibility to hypoxia-induced apoptosis. We exposed trophoblasts to 24-h intervals of hypoxia at different time intervals during 72 h in culture. Exposure of trophoblasts to hypoxia early in culture (0–24 h) resulted in enhanced apoptosis, compared with hypoxia during a later interval (48–72 h) in culture (Fig. 6), further supporting the premise that less differentiated trophoblasts in culture are more susceptible to the effect of hypoxia, compared with more differentiated trophoblasts. Additionally, preexposure of trophoblasts to EGF for 24 h before an additional 24 h in hypoxia also decreased the level of apoptosis by 0.5 ± 0.1-fold (P < 0.05), compared with cells that were not preexposed to EGF (Fig. 7).

**DISCUSSION**

Our study demonstrates that hypoxia enhances apoptosis in cultured term human trophoblasts, as assessed by DNA fragmentation, morphologic changes consistent with apoptosis, and the reversibility of the apoptotic process by a caspase inhibitor. The apoptotic process is accompanied by an increase in the expression of p53 and a change in the ratio of the proapoptotic Bax and the antiapoptotic Bcl-2. EGF inhibited much of the apoptosis induced by hypoxia, suggesting differentiation of trophoblasts may confer resistance to hypoxia.

Hypoxia triggers apoptosis in a number of cell systems (3, 10, 19). The mechanism by which hypoxia induces apoptosis is postulated to involve mitochondrial pathways, as opposed to ligand-receptor stimuli mediated by cytokines such as TNF-α or Fas ligand. In the former pathway, stimulation of death signals occurs through modulation of the expression of specific genes such as p53 and members of the Bcl-2 family (7). Our study demonstrates an increased expression of the protein product of tumor suppressor gene p53 in cultured trophoblasts exposed to hypoxia, and this increased p53 expression correlates with enhanced cell death in trophoblasts. The p53 protein plays a pivotal role in the cellular response to DNA damage, specifically halting the cell cycle and allowing repair of DNA. If repair is not possible, p53 promotes apoptosis (14). The p53 is an unstable protein with a short half-life, but exogenous stimuli such as hypoxia and oxidative stress stabilize the p53 protein (2). The stable p53 protein...
plays a role in hypoxia-induced cell death in neurons (3), cardiomyocytes (16), and endothelial cells (28).

The mechanism by which p53 induces apoptosis is incompletely understood. The p53 protein upregulates the expression of Bax and downregulates Bcl-2 protein expression in different tissues during cell apoptosis (17). Conversely, others have found an increase in Bax expression, which acts downstream of p53, with no change in Bcl-2 expression (32). We found that in cultured trophoblasts, the increase in p53 expression in response to hypoxia coincided with the increase in Bax and the decrease in Bcl-2 expression.

The Bcl-2 family of proteins plays a major role in the regulation of the apoptotic processes (13). Hetero- and homodimers of the proapoptotic Bax and the antiapoptotic Bcl-2 determine cell survival or death by affecting the permeability of the mitochondrial membrane (21). Although we found an increase in Bax expression and a decrease in Bcl-2 expression in trophoblasts exposed to hypoxia, the concentration of Bax in trophoblasts maintained 24 h in culture was ~15-fold higher than that of the Bcl-2 protein. Thus Bax homodimers likely play a major role in the apoptotic process in trophoblasts. Others have found that Bax is translocated from the cytosol to the mitochondria during hypoxia (25), and this results in an increase in mitochondrial permeability, release of cytochrome c, and initiation of the apoptotic sequence. Additional experiments in which p53 and Bax are overexpressed or inhibited are necessary to further clarify the role of these proteins in mediating hypoxia-induced apoptosis in trophoblasts.

Our results also demonstrate that EGF diminishes the degree of apoptosis induced by hypoxia in cultured term trophoblasts. EGF plays an important role in differentiation and peptide hormone secretion in term placenta (18). Deficiency of EGF causes intrauterine growth restriction in mice (11), and placentas from pregnancies complicated by FGR exhibit a reduced number and an impairment of the EGF receptors, which may contribute to the placental dysfunction characteristic of these pregnancies (8). EGF suppresses apoptosis in other tissues (23, 30) and attenuates the apoptosis induced by the ligand-receptor pathway activated by the cytokines TNF-α and interferon-γ in cultured trophoblasts (9). Collectively, these data suggest an important role for EGF in protecting the integrity of the placenta against endogenous and exogenous stress. Because EGF inhibits apoptosis induced through both the ligand-receptor pathway and the mitochondria-mediated pathway, the effect of EGF most likely involves downstream effectors common to both pathways in the apoptotic cascade. Moreover, we speculate that the effect of EGF on apoptosis in trophoblasts may play an important role in protecting the placenta from hypoxic injury in pregnancies complicated by placental hypoperfusion.

Three observations in our study support the premise that the differentiated trophoblast phenotype, i.e., spongiotrophoblasts, are more resistant to hypoxia-induced apoptosis than the less differentiated cytotrophoblast phenotype: 1) we found that cell death occurred primarily in the first 24 h in culture, when most of the cells are undifferentiated cytrophoblasts (20, 21) hypoxia is known to hinder differentiation in cultured trophoblasts (20) while enhancing the expression of p53 and Bax, two proapoptotic proteins that predominate in cytrophoblasts (24), and 3) with the use of two culture conditions (differentiation in medium 199 for 48 h an addition of EGF), we demonstrated that the differentiated trophoblast phenotype is more resistant to hypoxia-induced apoptosis, compared with the undifferentiated cytrophoblast phenotype. The relative resistance of the spongiotrophoblast phenotype to acute and extreme hypoxia may be a defense mechanism by which this crucial cell layer maintains critical functions even in the presence of extreme environmental stress. Further experiments are needed to test this hypothesis.

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