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Hypoxia-Induced Downregulation of XIAP in Trophoblasts Mediates Apoptosis Via Interaction With IMUP-2: Implications for Placental Development During Pre-Eclampsia

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ABSTRACT

The regulation of trophoblast apoptosis is essential for normal placentation, and increased placental trophoblast cell apoptosis is the cause of pathologies such as intrauterine growth retardation (IUGR) and pre-eclampsia. X-linked inhibitor of apoptosis (XIAP) is expressed in trophoblasts, but little is known about the role of XIAP in placental development. In the present study, the function of XIAP in the placenta and in HTR-8/SVneo trophoblasts under hypoxic conditions was examined. In addition, the correlation between XIAP and immortalization-upregulated protein-2 (IMUP-2) was demonstrated in HTR-8/SVneo trophoblasts under hypoxia, based on a previous study showing that increased IMUP-2 induces trophoblast apoptosis and pre-eclampsia. XIAP was downregulated in pre-eclamptic placentas ($P < 0.05$). In HTR-8/SVneo trophoblasts, XIAP expression was decreased and the expression of apoptosis-related genes was increased in response to hypoxia. Ectopic expression of hypoxia inducible factor (HIF)-1α in HRT-8 SV/neo cells induced the nuclear translocation of XIAP and alterations of XIAP protein stability. Furthermore, hypoxia induced nuclear translocated XIAP co-localized with upregulated IMUP-2 in trophoblast nuclei, and the interaction between XIAP and IMUP-2 induced apoptosis in HRT-8 SV/neo cells. The present results suggest that hypoxia-induced down-regulation of XIAP mediates apoptosis in trophoblasts through interaction with increased IMUP-2, and that this mechanism underlies the pathogenesis of pre-eclampsia. J. Cell. Biochem. 114: 89–98, 2013. © 2012 Wiley Periodicals, Inc.

KEY WORDS: X-LINKED INHIBITOR OF APOPTOSIS; IMMORTALIZATION-UPREGULATED PROTEIN-2; PRE-ECLAMPSIA; TROPHOBLAST; HYPOXIA; APOPTOSIS

Placental development is essential for normal fetal growth, and requires the precise regulation of cellular events such as proliferation, differentiation and death [Kingdom et al., 2000; Lunghi et al., 2007]. During the first trimester of pregnancy, successful invasion of extravillous trophoblasts into the maternal placental bed is essential for proper fetal development. Invading extravillous trophoblasts dilate the utero-placental spiral arteries, facilitating the transport of metabolites, gases, waste, and nutrients

Su Yeon Jeon and Hyun-Jung Lee contributed equally to this work.

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between the mother and fetus [Moll et al., 2007]. During development, placental villous cytotrophoblasts undergo differentiation and fusion to form the syncytiotrophoblast. Regeneration of the villous syncytiotrophoblast is offset by apoptosis, which results in the extrusion of accumulated apoptotic nuclei (syncytial knots) into the maternal circulation [Huppertz et al., 1998]. Abnormal placental development due to inadequate trophoblast function leads to a number of obstetric diseases, including intrauterine growth retardation (IUGR), pre-term labor (PTL) and pre-eclampsia [Huppertz, 2006].

Pre-eclampsia is a pregnancy-associated disorder that is characterized by the onset of hypertension, proteinuria, and general edema after 20 weeks of gestation. It is a serious obstetric disease, and is associated with morbidity and mortality of both the mother and fetus [Noris et al., 2005]. Pre-eclampsia is associated with reduced placental perfusion and villous hypoxia [Levy and Nelson, 2000]. Hypoxia disturbs normal placentation through the induction of apoptosis in cytotrophoblast and syncytiotrophoblast cells [Ishihara et al., 2002; Hu et al., 2006] and through the stimulation of syncytial knot formation [Hung et al., 2008]. While several risk factors for pre-eclampsia have been reported, the balance between proliferation and apoptosis in trophoblasts, the regulation of which is required for normal placentation, has yet to be explored as a factor in the pathogenesis of pre-eclampsia. We previously reported that the hypoxia-induced increase in immortalization-upregulated protein-2 (IMUP-2) induces apoptosis of trophoblasts, and suggested that increased IMUP-2 could be used as a marker for pre-eclampsia based on the functional involvement of IMUP-2 in placental development and gynecological diseases [Jeon et al., 2010].

Inhibitor of apoptosis proteins (IAPs) are part of one of the most powerful intrinsic pathways of apoptosis inhibition [Holcik and Korneluk, 2001]. To date, eight human IAPs have been identified, of which XIAP is the most potent and versatile. XIAP contains three baculoviral IAP repeat (BIR) domains and a C-terminal Zinc finger/RING domain, and has been shown to differentially inhibit caspase activity [Suzuki et al., 2001; Verhagen et al., 2001]. XIAP is expressed in trophoblasts, and its levels significantly increase during the first trimester, during which cytotrophoblasts differentiate and fuse to form the syncytiotrophoblast [Gruslin et al., 2001]. The role of XIAP in normal or pre-eclamptic placental development has not been elucidated to date.

In the present study, the expression of XIAP in normal and pre-eclamptic placentas and the function of XIAP in HTR-8/SVneo trophoblasts under hypoxic conditions were analyzed. Furthermore, the correlation between XIAP and immortalization-upregulated protein-2 (IMUP-2) was demonstrated based on a previous report that increased IMUP-2 induces trophoblast apoptosis and pre-eclampsia.

MATERIALS AND METHODS

PLACENTA SAMPLE COLLECTION

Placentas were collected from normal subjects showing no evidence of medical, obstetric or surgical complications who underwent a term delivery ($\geq$37 gestational weeks). Pre-eclampsia was defined as the presence of hypertension (systolic blood pressure of 140 mmHg or diastolic blood pressure of 90 mmHg on at least two occasions), proteinuria (2+ protein by urine dipstick or higher than 300 mg in a 24-h urine collection), and edema. Placental tissues were collected from the following groups of patients: (1) term without labor ($n = 15$); (2) term without labor pre-eclampsia ($n = 15$); and (3) pre-term without labor pre-eclampsia ($n = 11$). Written, informed consent was obtained from all patients prior to the collection of samples. The collection and use of the samples for research purposes was approved by the Institutional Review Board of CHA General Hospital, Seoul, Korea.

CELL CULTURE AND HYPOXIA

HTR-8/SVneo cells were cultured in RPMI 1640 medium (Gibco, Logan, UT) supplemented with 5% fetal bovine serum (FBS; Gibco) and 100 U/ml penicillin and streptomycin (Gibco). To increase the efficiency of the hypoxia treatment, the medium was exposed to hypoxia for 24 h before use (hypoxic medium). Six hours after seeding, HTR-8/SVneo cells were switched to the hypoxic medium, and then exposed to hypoxic conditions (1% O_2, 99% N_2) for different periods of time at 37°C. Cells were washed with phosphate buffered saline (PBS) and treated with 0.05% trypsin-EDTA (Gibco) at 37°C for 2 min. Cells were collected by centrifugation and stored at −80°C until use.

EXPRESSION OF HIF-1A IN HTR-8/SVNEO CELLS

HTR-8/SVneo cells were transfected with an expression vector for HIF-1α using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Briefly, HTR-8/SVneo cells at approximately 70% confluence were transfected with 10 μg of an expression plasmid for HIF-1α for 6, 12, 24, and 48 h. Transfected cells were harvested and analyzed.

KNOCK-DOWN EXPRESSION OF HIF-1A AND XIAP IN HTR-8/SVNEO CELLS USING siRNA

To induce knock-down expression of HIF-1a and XIAP, transfection experiments were performed with HTR8/SV-Neo cells. They were cultured at 37°C and 5% CO_2 in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (Invitrogen), 1% penicillin/streptomycin (GIBCO, Invitrogen, Grand Island, NY), prior to transfection which was carried out in Opti-Mem. HTR-8/SVneo cells were cultured at 50–60% confluence and transfected with the HIF-1a siRNA duplex (siRNA-HIF-1a) and XIAP siRNA duplex (siRNA-XIAP) or a negative control (Mirus Bio LLC, WI). The HIF-1a specific target sequences used in the study were 5′-AGUUAGUUCAAACUGAGUUAAUCCCUU-3′ (sense) and 5′-GGGAUUAACUCAGUUUGAACUAACUUU-3′ (antisense). The XIAP specific target sequences used in the study were 5′-GCAGAUUAUGAAGCACGGAUCUUTA-3′ (sense) and 5′-UAAAGAUCCGUGCUUCAUAAUCUGCCA-3′ (antisense). Cells were transfected with siRNA-HIF-1a or siRNA-XIAP using Lipofectamine 2000, according to the manufacturer's instructions. The concentrations of used siRNA in HIF-1a and XIAP were used 20 and 200 pM into 100 mm dish and the dishes were incubated for 48 h.

FACS ANALYSIS

For FACS analysis, HTR-8/SVneo cells were dissociated with a cell dissociation buffer (GIBCO). HTR-8/SVneo cells viabilities depend on hypoxia treatment (6, 12, and 24 h) was measured the sub G1 fraction using propidium iodide (PI; 5 ng/ml, Sigma) used to identify nonviable cells. Stained cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, NJ). For each sample, at least 10,000 events were acquired.

SEMIQUANTITATIVE REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION (RT-PCR)

Frozen placental tissues were ground in liquid nitrogen and total RNA was extracted using TRIzol (Invitrogen). Total cellular RNA from 1% hypoxia-treated and HIF-1α-overexpressing HTR-8/SVneo cells was extracted using RNeasy Mini Kits (Qiagen, Valencia, CA). Reverse transcription was carried out using 1 μg of purified total RNA and Superscript III reverse transcriptase (Invitrogen), and the synthesized cDNAs were amplified by PCR. The primers used for RT-PCR are presented in Table I. The thermal cycling parameters were as follows: initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at the appropriate temperature for each set of primers for 40 s, and extension at 72°C for 1 min. Amplified products were stained using ethidium bromide solution (0.5 μg/ml), and then visualized using a video image analyzer (Bio-Rad, Hercules, CA). The genes of interest, and 28S rRNA as a control, were quantified by densitometry using Quantity One software (Bio-Rad). Data were presented as the ratio of the optical density of the gene of interest to that of 28S rRNA.

WESTERN BLOT ANALYSIS

Placental tissue was homogenized in protein extraction solution (Intron, Kyunggi, South Korea). Lysates were subjected to sonication for 20 s, followed by centrifugation at 12,000 rpm for 15 min at 4°C. Total protein concentration in the supernatants was determined using a BCA protein assay kit (Pierce, Rockford, IL). After heating at 95°C for 5 min, total protein extract (80 μg) was resolved by 10% SDS–polyacrylamide gel electrophoresis (PAGE). Proteins were transferred onto a PVDF membrane at 90 V for 100 min, and then membranes were stained with Ponceau S solution (Sigma, MO) to confirm transfer. Membranes were incubated with 5% nonfat dry milk in TBS-T (TBS with 0.05% Tween 20) for 60 min at room temperature. The membranes were then incubated with one of the following primary antibodies, as indicated: anti-XIAP (1:2,000; R&D systems, Minneapolis, MN), anti-Bak (1:1,000; EPITOMICS), anti-caspase-3 (1:500; Asp175, 5A1E, Cell Signaling Technology), anti-caspase-9 (1:4,000; R&D systems), anti-Bcl-2 (1:500; Santa Cruz Biotechnology), anti-HIF-1α (1:1,000), or anti-β-actin (1:5,000; Santa Cruz Biotechnology) at 4°C overnight, followed by incubation with horseradish peroxidase (HRP)-conjugated goat (1:10,000), rabbit (1:10,000) or mouse (1:10,000) secondary antibody. Immunoreactive proteins were visualized using ECL or ECL Advanced Western Blotting detection kits (Amersham, Piscataway, NJ).

TABLE I. Primer Sequences for RT-PCR Analysis in the Study Used

Name	Sequence (5′ → 3′)
XIAP	GTGACTAGATGTCCACAAGG CTTGAGGAGTGTCTGGTAAG
BIR-1	GAGTTTAATAGATTAAAA ACTTTTG ATTTGGGGATACTTTCCTGTGTCTTC
BIR-2	GAGAATTCAGAGATCATTTTGCCTTAGACAGG CTGGAAAGCTTATTCACTTCGAATATTAAGATTCC
BIR-3	CGA ATTCTCTGATGCTGTGAGTTCTGATAG GTACGAAGCTTAAGTAGTTCTTACCAGACACTCC
Bak	CCAGGTCCTCCCAGGCAG CCCAGA ACCACCAGCACG
Caspase-3	CAGAACTGGACTGTGGCA TAGCGTCAAAGGAAAAGG
Caspase-9	GAACTTCTGCCGTGAGTC GGGTTACTGCCAGGGGAC
Bcl-2	CCGAGATGTCCAGCCAGC GCCAAACTGAGCAGAGTC
IMUP-2	ATGGAGTTCGACCTGGG ACTTCACATCCGTGTCCG
28S rRNA	TTGAAAATCCGGGGGAGAG ACATTGTTCCAACATGCCAG

NUCLEAR AND CYTOPLASMIC CELL FRACTIONATION

Nuclear and cytoplasmic fractions from hypoxia-treated cells and from HIF-1α-overexpressing cells were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce), according to the manufacturer's instructions. Briefly, cells were harvested and suspended in 200 μl of ice-cold CERI buffer containing a protease inhibitor cocktail tablet (Roche, Indianapolis, IN). Cells were incubated on ice for 10 min, after which 11 μl of ice-cold CERII buffer was added. The mixture was vortexed and incubated on ice for 1 min. After centrifugation, the supernatant (cytoplasmic fraction) was collected, and the insoluble pellet, containing the nuclei, was resuspended in 100 μl of ice-cold NER buffer containing a protease inhibitor cocktail tablet. After incubation on ice for 40 min, the supernatant (nuclear fraction) was collected by microcentrifugation. Nuclear and cytoplasmic fractions (20 μl) were resolved by SDS–PAGE and analyzed by Western blotting.

IMMUNOHISTOCHEMISTRY

Placental tissue was obtained from the central and peripheral regions of placentas, fixed in 10% neutral buffered formaline (10% NBF), and then embedded in paraffin wax. Paraffin-embedded tissue was cut into 8-μm thick sections, and the sections were de-waxed and rehydrated with a graded ethanol series. Sections were immersed in a proteinase K solution (Dako, Carpinteria, CA) for 5 min, and the slides were incubated for 30 min at room temperature in blocking solution (Dako) to block nonspecific binding, followed by incubation with anti XIAP (R&D systems; 1:200) at 4°C overnight in a humidified chamber. After washing with PBS, the slides were immersed in 3% hydrogen peroxide to inhibit endogenous peroxidase activity, and then incubated with biotinylated link universal solution (Dako) for 1 h in a humidified chamber. Following incubation with streptavidin-HRP (Dako), immunoreactive proteins were detected using 3,3-diaminobenzidine tetrahydrochloride (DAB) substrate-chromogen solution. Slides were counterstained with Mayer's hematoxylin and mounted onto coverslips using a mounting solution.

IMMUNOFLUORESCENCE

The expression of XIAP was detected on hypoxia-treated and HIF-1α-overexpressing HTR-8/SVneo cells by immunofluorescence. The cells were blocked with normal blocking serum for 30 min, and incubated with anti-XIAP (1:200) at 4°C overnight. After further incubation with Alexa Fluor 488 chicken anti-goat IgG (1:400, Molecular Probes, Inc., Eugene, OR) for 1 h, the stained slides were mounted with a glycerol-based mounting solution containing 1, 4-diazabicyclo-octane with PI (Sigma, St. Louis, MO). All images were acquired using an LSM 510 META confocal microscope (Carl Zeiss, Inc., Switzerland).

For double immunofluorescence, cells under normoxia or hypoxia were processed as describe above. Cells were incubated with anti-IMUP-2 (1:50) at 4°C overnight and anti-XIAP (1:200; R&D systems) at room temperature for 2 h and subsequently with FITC-conjugated chicken anti-rabbit secondary antibody (1:200; Molecular Probes, Inc.) or Rhodamine-conjugated chicken anti-goat secondary antibody (1:400; Molecular Probes, Inc) for 1 h at room temperature. Cells were then washed, and stained with DAPI (30 nM) for 3 min at room temperature prior to mounting on glass slides. For negative controls, the appropriate non-immune IgG was used instead of primary antibodies. Double-immunofluorescence was visualized using an LSM 510 META confocal microscope (Carl Zeiss, Inc.).

IMMUNOPRECIPITATION

Total cellular extracts were prepared from cells exposed to hypoxic conditions or normoxia using a protein extraction solution (Intron) followed by sonication for 20 s and by centrifugation at 13,000 rpm for 15 min at 4°C. A 500 μg aliquot of total proteins was subjected to immunoprecipitation followed by Western blotting according to the manufacturer's instructions. Briefly, the cell lysates were incubated with 2 μg of anti-IMUP-2 and/or anti-XIAP (R&D systems) at 4°C for 2 h, followed by 50 μl of protein A/G-Sepharose beads (Amersham) at 4°C overnight with constant shaking. Negative controls were performed under identical conditions by incubating with equal amounts of non-immune IgG instead of the primary antibody. Immunoprecipitates were washed twice with cell lysis buffer and once with PBS. Bound proteins were eluted by boiling and subjected to Western blotting for anti-XIAP (1:2,000; R&D systems) and/or anti-IMUP-2 (1:1,000).

STATISTICS

RT-PCR data were analyzed by *t*-test to determine differences in gene expression between normal, second-trimester pre-eclamptic and third-trimester pre-eclamptic placentas. A P value of <0.05 was considered statistically significant.

RESULTS

The expression of XIAP in placental tissues was confirmed by RT-PCR analysis in different regions (e.g., central and peripheral regions) of normal placentas, as well as in placentas from patients with second- or third-trimester onset pre-eclampsia, and gene expression levels were normalized to those of 28S rRNA used as the internal control. XIAP was strongly expressed in the normal placenta, while its expression was decreased in second- and third-trimester onset pre-eclamptic placentas (Fig. 1A, $P < 0.001$). Also, the levels of the BIR-1, BIR-2, and BIR-3 domains of XIAP were also significantly higher in normal placentas than in pre-eclamptic placentas (Fig. 1B–D, $P < 0.05$). These results indicate that the expressions of XIAP and other domains of XIAP such as BIR-1, BIR-2, and BIR-3 decreased in pre-eclamptic placentas compared to normal placentas. Immunohistochemistry was used to further determine the localization and expression pattern of placental XIAP. XIAP was detected in syncytiotrophoblasts and syncytial knots. Prominent XIAP protein expression was detected in the peripheral region of normal placentas, whereas XIAP levels were decreased in all regions including central and peripheral regions of second-trimester pre-eclamptic placentas (Fig. 2A). In addition to the expression of XIAP in the placenta, the relationship between XIAP expression and that of apoptosis-related factors in normal and pre-eclamptic placentas was analyzed based on Western blot results showing an association between pre-eclampsia and trophoblast apoptosis in response to hypoxic conditions (Fig. 2B). The expression of XIAP and HIF-1α was significantly lower in the central region of second-trimester pre-eclamptic placentas as compared to normal placentas. By contrast, the expression of several apoptosis-related factors, including caspase-3, caspase-9 and bcl-2, differed between normal and second-trimester pre-eclamptic placentas in the peripheral region. Consistent with the decreased expression of XIAP, the levels of the active form of caspase-3 were increased in pre-eclamptic placentas, particularly during the second-trimester. The expression of HIF-1α in the peripheral placental region was also increased in second-trimester pre-eclamptic placentas. These results indicated that the hypoxia-mediated reduction of XIAP expression plays a role in abnormal placentation, and in particular, in pre-eclampsia.

To determine whether decreased XIAP expression in pre-eclamptic placentas triggered trophoblast apoptosis, the apoptosis of HTR-8/SVneo cells was analyzed under conditions of hypoxia by RT-PCR, Western blotting and immunofluorescence analysis. As shown in Figure 3, following hypoxic challenge, HTR-8/SVneo cells exhibited marked morphological changes that were indicative of apoptosis (Fig. 3A, arrow), and showed a significant increase in the rate of cell death (Fig. 3B, $P < 0.05$). Under hypoxic conditions, the mRNA levels of XIAP, BIR-2, and BIR-3 in HTR-8/SVneo cells were significantly reduced, whereas those of the apoptosis-related factors Bak, caspase-3, and caspase-9 were increased compared to control levels (Fig. 3C, $P < 0.05$). There were no differences in the expression of BIR-1 and Bcl-2 at any point following exposure to hypoxia. Western blot analysis confirmed that the expression of the active form of XIAP (54 kDa) was noticeably reduced after 12 h of hypoxia (Fig. 3D), consistent with the results of mRNA analysis. In addition, the expression of Bcl-2 was decreased; otherwise, the expressions of caspase-3 and caspase-9 were increased in trophoblasts under hypoxic conditions.

Because XIAP localizes to the nucleus and the cytoplasm, and its function is believed to be dependent on its subcellular localization, immunocytochemistry was used to examine the subcellular localization of XIAP in response to hypoxia in HTR-8/SVneo

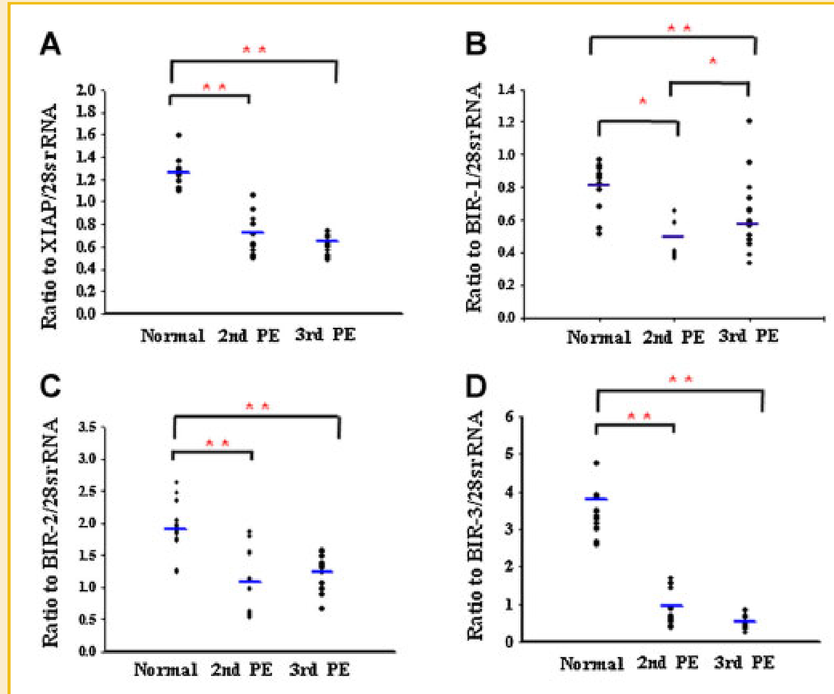

Fig. 1. mRNA expression of XIAP in normal and pre-eclampsia placentas. Densitometric analysis of XIAP mRNA expression (A), BIR-1 domain of XIAP mRNA expression (B), BIR-2 domain of XIAP mRNA expression (C), and BIR-3 domain of XIAP mRNA expression (D) relative to 28S rRNA mRNA expression. (N, normal placenta; PE, pre-eclampsia placenta. 2nd PE, second trimester pre-eclampsia; 3rd PE, third trimester pre-eclampsia). Data are expressed as means $\pm$ SD. Statistical analysis was carried out by unpaired Student's *t*-tests ($^{*}P < 0.05$, $^{**}P < 0.001$).

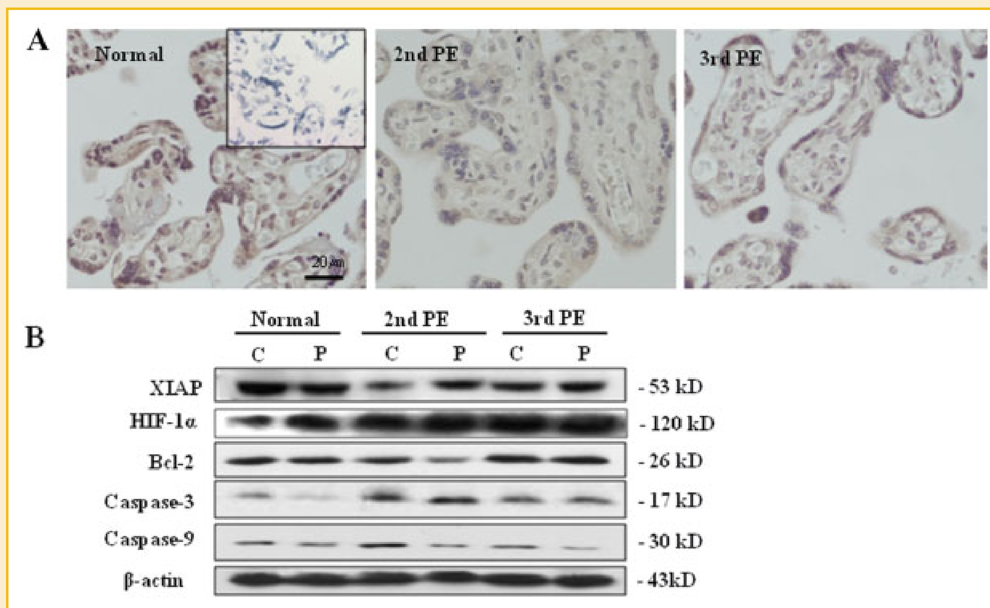

Fig. 2. Expression of XIAP in normal and pre-eclampsia placentas. A: XIAP expression was analyzed in normal and pre-eclamptic placental sections by immunohistochemistry (upper part: negative control in normal placenta; $\times 200$). B: Western blot analysis of XIAP, HIF-1α, caspase-9, caspase-3, and Bcl-2. β-actin was used as a loading control for placental protein (N, normal placenta; PE, pre-eclampsia placenta. 2nd PE, second trimester pre-eclampsia; 3rd PE, third trimester pre-eclampsia; C, central region; P, pheripheral region).

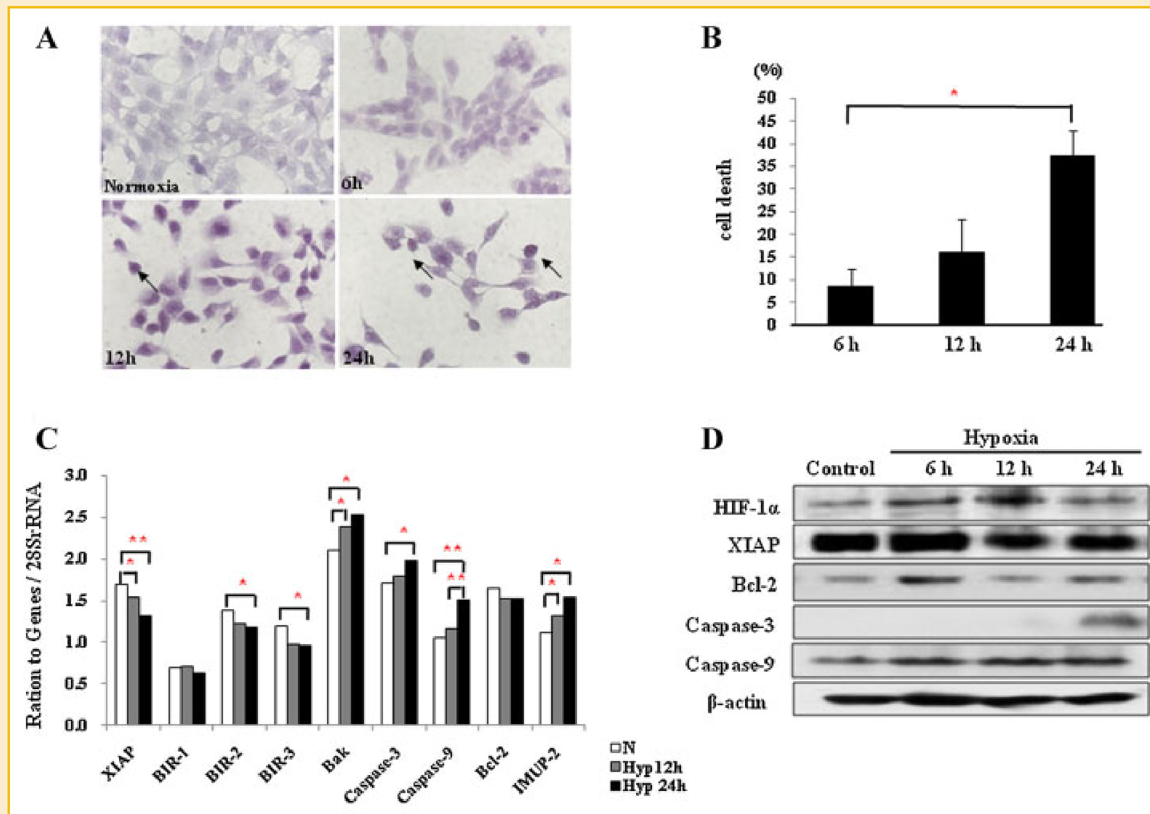

Fig. 3. Decreased XIAP expression induces apoptosis in HTR-8 SV/neo trophoblasts under hypoxic conditions. A: Trophoblasts exhibited morphological changes consistent with apoptosis following exposure to hypoxia (×200, arrow). B: Trophoblasts were exposed to hypoxia (1% O_2) for 6, 12, and 24 h, and cell death relative to untreated control cells was measured. C: The mRNA expression of XIAP was decreased and that of apoptosis-related genes was increased in cells exposed to hypoxia. D: Western blot analysis of XIAP and apoptosis-related genes following 24 h of hypoxia. Data are expressed as means ± SD. Statistical analysis was carried out by unpaired Student's *t*-tests ($^{*}P < 0.05$).

trophoblasts. Under conditions of normoxia, XIAP was detected predominantly in the cytoplasm (Fig. 4A). However, following exposure to hypoxia for 12 and 24 h, XIAP localized primarily to the nucleus. After 24 h of hypoxia, XIAP was evenly distributed in the cytoplasm and the nucleus. These results indicated that XIAP is translocated to and remains in the nucleus under hypoxic conditions, although some portion of XIAP returns to the cytoplasm. Subcellular fractionation and Western blot analysis (Fig. 4B) showed similar results, namely the detection of XIAP in the cytoplasmic fraction in normoxic cells, and its detection in nuclear fractions after 12 or 24 h of hypoxia. Moreover, the level of the cleaved form of XIAP (30 kDa), which is the inactive form of XIAP, increased significantly under hypoxic conditions in a time-dependent manner. After 48 h of hypoxia, both the active and inactive forms of XIAP were selectively decreased in the nucleus (data not shown).

The possible involvement of HIF-1α, which is a key transcription factor in hypoxia-induced gene regulation, in the oxygen-dependent expression of XIAP was investigated. The half-life of HIF-1α was very short, and the protein was rapidly degraded after induction of hypoxic conditions. Due to these technical limitations, the role of HIF-1α in the regulation of XIAP expression and apoptosis was examined using a transient transfection system. HTR-8/SVneo trophoblasts were transfected with an expression plasmid for HIF-1α, and the expression of XIAP and induction of apoptosis were measured 6, 12, and 24 h after transfection. As shown in Figure 5, there was an increase in apoptotic cell morphology and cell death (approximately 20% decrease) in HIF-1α-overexpressing cells as compared to controls (Fig. 5A). Accordingly, the expression of XIAP was down-regulated and the level of activated caspase-3 was increased in HIF-1 α-overexpressing cells (Fig. 5B). In addition, we analyzed the expression of XIAP in trophoblasts by using siRNA against HIF-1α in HTR-8SV/neo trophoblast cells to confirm the correlation between HIF-1α and XIAP. As shown in Figure 5C,D, the expression of HIF-1α was down-regulated after siRNA treatment for HIF-1α, and the expressions of XIAP and BCl_2 were slightly increased. The expression of caspase-9 was decreased. Also, we performed XIAP siRNA to evaluate the function of XIAP in apoptosis. siRNA mediated down-regulation of XIAP induced apoptosis in trophoblast cells (Fig. 5C,D). These findings suggest that increased HIF-1α in trophoblast cells under hypoxic conditions induce apoptosis through the down-regulation of XIAP.

A previous study reported that the hypoxia-induced increase in IMUP-2 induces trophoblast apoptosis and pre-eclampsia [Jeon et al., 2010]. Therefore, the cellular distribution of XIAP and its potential interaction with IMUP-2 were investigated by double immunofluorescence. Under normoxia, the immunofluorescence

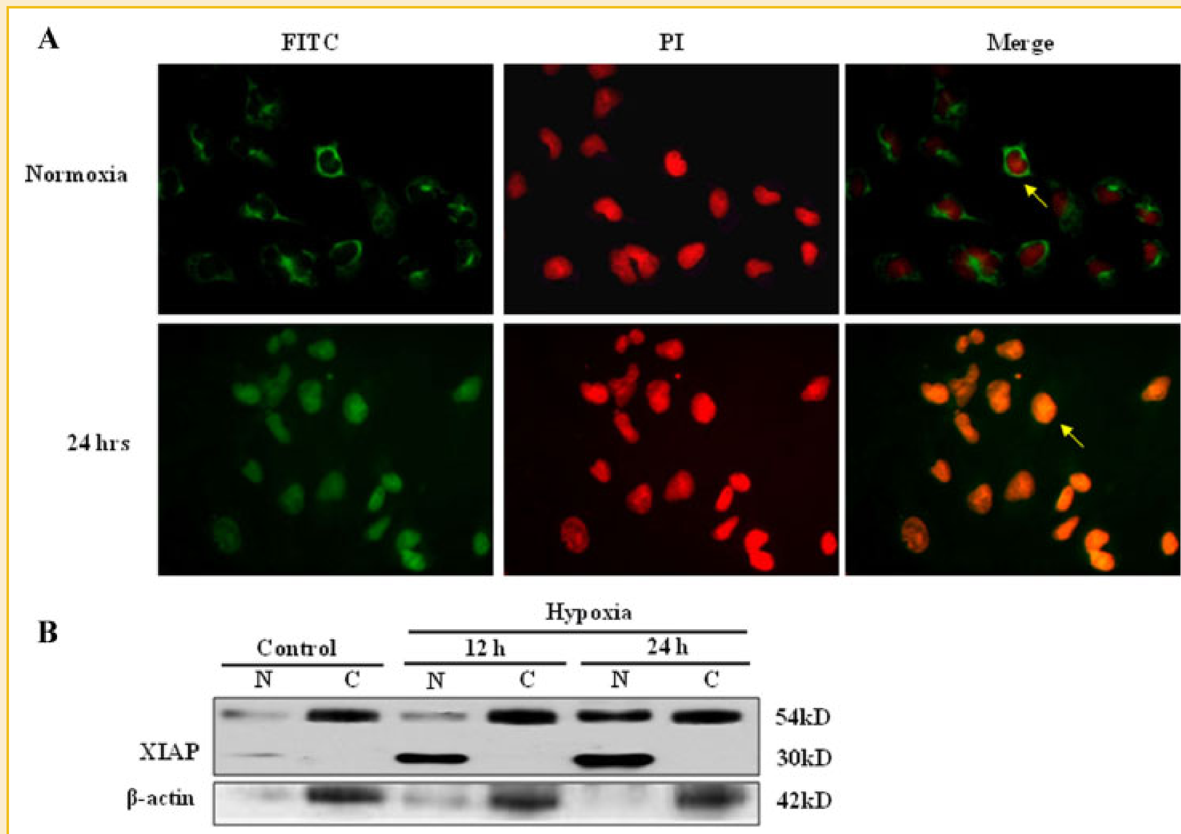

Fig. 4. XIAP translocates from the cytoplasm to the nucleus in response to hypoxia. A: Cellular location of XIAP in HTR-8 SV/neo trophoblasts exposed to hypoxia using immunofluorescence (×200). B: The separation of cytoplasmic and nuclear fractions for XIAP expression in HTR-8 SV/neo trophoblasts exposed to hypoxic condition was confirmed by Western blot analysis. β-actin was used as a loading control for the cellular fractionation. PI was used for nuclear staining.

corresponding to IMUP-2 (green) was localized to the nucleus and the XIAP-specific immunofluorescence (red) was mostly localized to the cytoplasm. The expression of IMUP-2 was significantly increased in cells under hypoxia compared with the control normoxia cells, and XIAP was translocated to the nucleus. Co-localization of IMUP-2 and XIAP was observed exclusively in the nucleus (Fig. 6A). To confirm whether HIF-1α was involved in the hypoxia-induced nuclear translocation of XIAP, the localization of XIAP was investigated in HIF-1α overexpressing trophoblasts. HTR-8/SVneo cells were transfected with an expression vector for HIF-1α, and the relocalization of XIAP was assessed after 48 h by Western blotting. XIAP relocalized to the nucleus and was detected in the nuclear fractions of HIF-1α-overexpressing cells (Fig. 6B). These results indicated that the nuclear translocation of XIAP in response to hypoxia is mediated by HIF-1α.

In addition, immunoprecipitation of total cellular proteins using anti-IMUP-2 and/or anti-XIAP antibodies followed by Western blot analysis revealed increased XIAP protein levels co-precipitated with IMUP-2 in hypoxia-exposed cells compared to normoxia controls. These results clearly showed an increased association between IMUP-2 and XIAP in trophoblast cells exposed to hypoxic conditions (Fig. 6C). These findings suggest that the interaction between nuclear translocated XIAP and increased IMUP-2 in trophoblast nuclei triggered by hypoxia can induce apoptosis in trophoblasts.

DISCUSSION

The current study demonstrated that XIAP is downregulated in syncytiotrophoblasts and syncytial knots of the placental villi in pre-eclamptic placentas, particularly during the second trimester. XIAP, which is normally localized to the cytoplasm, was translocated to the nucleus in HTR-8/SVneo trophoblasts in response to hypoxia, where it interacted with IMUP-2, which was increased under hypoxic conditions. Finally, increased IMUP-2 expression and hypoxia-induced translocation of XIAP were associated with trophoblast cell death, which indicates that the subcellular distribution of XIAP and binding with IMUP-2 play a crucial role in the regulation of trophoblast cell death under hypoxic conditions.

During normal placentation, villous trophoblasts invade the endothelium of the uterus and remodel maternal uterine spiral arteries to increase utero-placental blood flow [Osol and Mandala, 2009]. Thus, trophoblast function and physiology (e.g., survival and apoptosis) are important in sustaining pregnancy. At this time, oxygen tension, as a key factor in trophoblast function, regulates

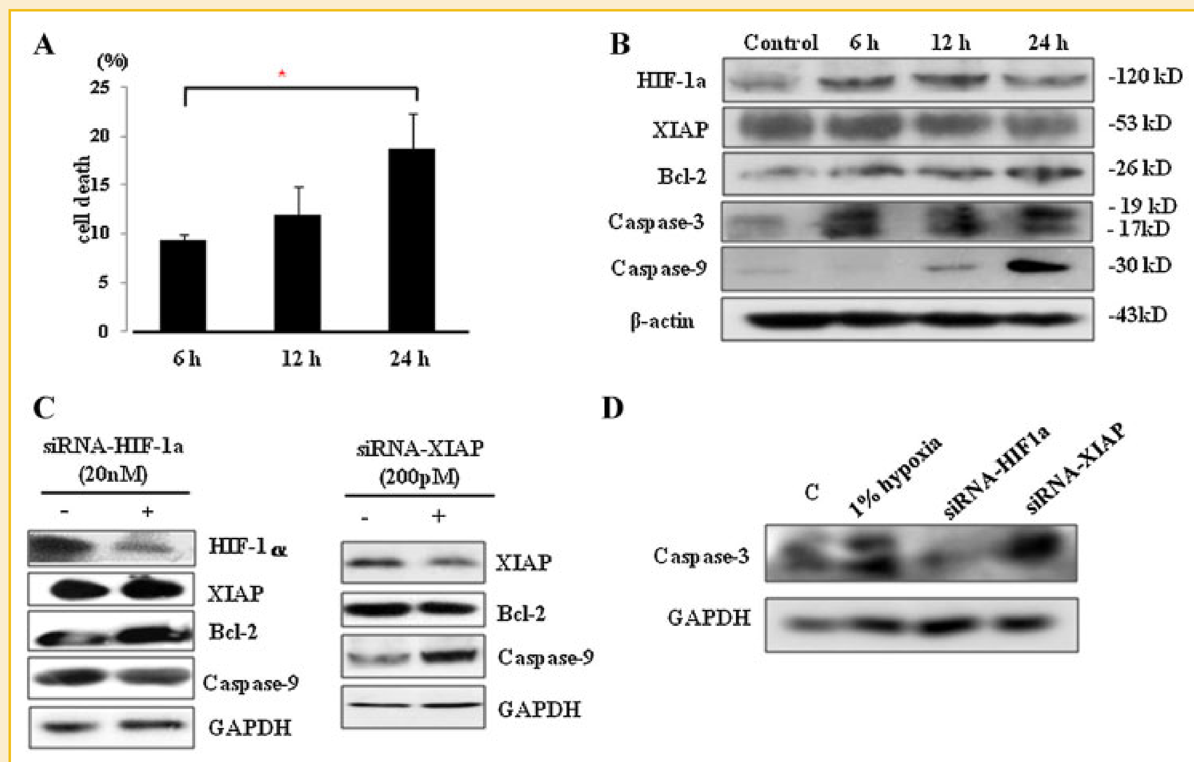

Fig. 5. Overexpression of HIF-1α induces apoptosis and inactivates XIAP in HTR-8 SV/neo trophoblasts transfected with an expression plasmid for HIF-1α. A: The rate of cell death of HTR-8 SV/neo trophoblasts after HIF-1α transfection. B: Western blot analysis for the expression of XIAP and apoptosis-related proteins. C: Western blot analysis for the expression of HIF-1a, XIAP, and apoptosis-related proteins depend on siRNA-HIF-1a and siRNA-XIAP treatment. D: caspase-3 expression in HTR-8SV/neo trophoblast after siRNA-HIF-1a and siRNA-XIAP treatment. Data are expressed as means ± SD. Statistical analysis was carried out by unpaired Student's *t*-tests ($^{*}P < 0.05$). β-actin and GAPDH were used as a loading control.

cell cycle and apoptosis in trophoblasts, and pre-eclampsia is induced under low oxygen (e.g., hypoxic condition) [Huppertz et al., 2006; Lyall, 2006]. Hypoxia inducible factors (HIFs) are transcription factors that are tightly regulated by oxygen tension, and play a crucial role in cellular responses to hypoxia [Taylor, 2008]. HIF-1α expression is increased in pre-eclamptic placental tissues [Caniggia and Winter, 2002; Rajakumar et al., 2008]. Our data are in agreement with these previous results, and show that HIF-1α is highly expressed in pre-eclamptic placentas as compared to normal placentas.

Post-mitochondrial mechanisms of cell survival involve the activity of several endogenous inhibitors of apoptosis, including IAPs and Bcl-2 [LaCasse et al., 1998]. XIAP is the most potent endogenous inhibitor of mammalian caspases, and functions by binding to and inhibiting both initiator and effector caspases [Hung et al., 2008]. XIAP contains three BIR domains (BIR-1, -2, and -3), which are believed to be essential for its caspase inhibitory function. Structurally and functionally, XIAP can be divided into an N-terminal region, which contains BIR1-2 and is cleaved into two fragments during apoptosis, and a second region that contains BIR-3 and the RING domain (BIR3-RING). The BIR-2 domain, together with the linker between BIR-1 and BIR-2, inhibits caspase-3 and caspase-7, whereas the BIR3-RING domain is a specific inhibitor of caspase-9 [Eckelman et al., 2006; Hung et al., 2008]. In real time PCR analysis, the expression of XIAP was decreased in the peripheral regions of pre-eclamptic placentas as compared to normal placentas (Supplement data 1). Based on this observation, we analyzed whether altered XIAP expression was associated with physiological changes in trophoblast cell function. Under hypoxic conditions, there was a significant decrease in XIAP expression and increased apoptosis in HTR-8/SVneo trophoblasts. We demonstrated that the expression of XIAP and BCl_2 in HTR-8/SVneo trophoblasts after siRNA-HIF-1a treatment was slightly increased; otherwise, siRNA mediated down-regulation of XIAP induced apoptosis in trophoblast cells (Supplement data 2). These results suggest that XIAP plays an important role in the regulation of placental apoptosis during placentation. Interestingly, the level of inactivated XIAP (30 kDa) was increased in the nucleus in response to hypoxia, but not by overexpression of HIF-1α. Thus, while HIF-1α mediates the nuclear translocation of XIAP, nuclear factor(s) other than HIF-1α appear to be involved in XIAP degradation. The regulatory mechanism and precise role of the cleaved form of XIAP will be the subject of future studies.

In a previous study, we found that the expression of immortalization-upregulated protein-2 (IMUP-2) in HTR-8/SV/neo trophoblast cells was increased under hypoxic conditions in correlation with an increase in trophoblast apoptosis and pre-eclampsia [Jeon et al., 2010]. Furthermore, increased expression of IMUP-2 and the subcellular redistribution of XIAP were associated with the co-

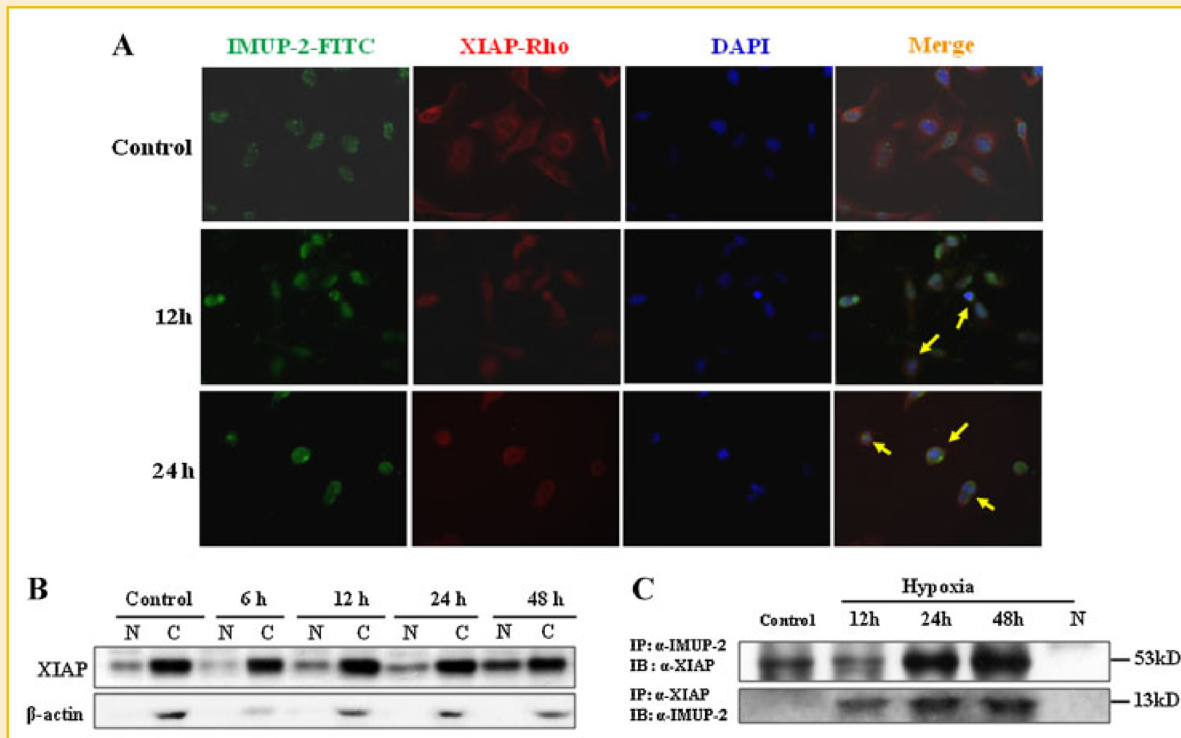

Fig. 6. Co-localization and interaction between XIAP and IMUP-2 in trophoblasts exposed to hypoxic condition. A: Co-localization of IMUP-2 (FITC, green) and XIAP (Rho, red) in the nucleus of trophoblast exposed to hypoxic condition using immunofluorescece (arrow). B: XIAP was translocated from the cytoplasm to the nucleus after HIF-1α transfection. C: Co-immunoprecipitation using anti-IMUP-2 and/or anti-XIAP in trophoblasts depends on hypoxia treatment. Increased IMUP-2/XIAP binding compared with normoxia control cells by Western blotting of specific immunoprecipitates with each antibody. N, negative control using non-immune IgG.

localization of these proteins in the nucleus under hypoxic conditions, and IMUP-2/XIAP binding increased in response to hypoxia in a time-dependent manner. Based on these findings, we suggested that the redistribution of XIAP to the nucleus may be correlated with IMUP-2 overexpression, and their binding could be related to trophoblast apoptosis; however, the underlying mechanisms remain unclear. The balance between XIAP and IMUP-2 in trophoblasts, as well as the interaction between these molecules, should be studied further to understand the precise mechanism of trophoblast apoptosis.

In conclusion, XIAP expression in syncytiotrophoblasts and syncytial knots was significantly decreased in pre-eclamptic placentas as compared to normal placentas, and decreased expression of XIAP was associated with increased apoptosis under hypoxic conditions. In cultured HTR-8/SVneo cells, nuclear translocation of XIAP was associated with trophoblast cell death in response to hypoxia, suggesting that cytoplasmic localization of XIAP may be essential for trophoblast survival. Moreover, the nuclear translocation of XIAP was associated with trophoblast cell death and nuclear XIAP interacted with IMUP-2 in response to hypoxia in HTR-8/SVneo trophoblast cells. These findings provide a better understanding of the function of IMUP-2 and XIAP in the placenta and the effect of hypoxia-induced down-regulation of XIAP in trophoblast apoptosis, and suggest that this mechanism may underlie the pathogenesis of pre-eclampsia.

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