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Increased Immortalization-Upregulated Protein 2 (IMUP-2) by Hypoxia Induces Apoptosis of the Trophoblast and Pre-eclampsia

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ABSTRACT

In regulation of the developmental process, the balance between cellular proliferation and cell death is critical. Placental development tightly controls this mechanism, and increased apoptosis of placental trophoblasts can cause a variety of gynecological diseases. Members of the immortalization-upregulated protein (IMUP) family are nuclear proteins implicated in SV40-mediated immortalization and cellular proliferation; however, the mechanisms by which their expression is regulated in placental development are still unknown. We compared IMUP-2 expression in normal and pre-eclamptic placental tissues and evaluated the function of IMUP-2 in HTR-8/SVneo trophoblast cells under hypoxic conditions. IMUP-2 was expressed in syncytiotrophoblasts and syncytial knots of the placental villi. IMUP-2 expression was significantly higher in preterm pre-eclampsia patients than in patients who went to term ($P < 0.001$); however, we observed no differences in IMUP-2 expression between normal term patients with and without pre-eclampsia. Hypoxic conditions increased apoptosis of HTR8/SVneo trophoblast cells and induced IMUP-2 expression. Also, apoptosis of HTR-8/SVneo trophoblast cells was increased after IMUP-2 gene transfection. These results suggest that IMUP-2 expression is specifically elevated in preterm pre-eclampsia and under hypoxic conditions, and that IMUP-2 induces apoptosis of the trophoblast. Therefore, IMUP-2 might have functional involvement in placental development and gynecological diseases such as pre-eclampsia. J. Cell. Biochem. 110: 522–530, 2010.

KEY WORDS: IMMORTALIZATION-UPREGULATED PROTEINS; PRE-ECLAMPSIA; TROPHOBLAST; HYPOXIA; APOPTOSIS

The fetal-derived placenta has neonatal and maternal components and plays a critical role as a feto-maternal organ during pregnancy. Support of fetal growth by transport of various metabolites between fetal and maternal blood is the most important function of the placenta. Therefore, placental dysfunction can result in severe damage to the developing fetus [Hemberger, 2007; Moore and Persaud, 2008].

Pre-eclampsia, a disorder characterized by onset of hypertension, proteinuria, and edema after 20 weeks of gestation, occurs in 5–10% of pregnancies and is one of the leading causes of maternal and neonatal morbidity and mortality [Sibai et al., 2003; Dekker and Sibai, 2008]. It also is associated with reduced placental perfusion and villous hypoxia [Levy et al., 2000]. Although the precise pathophysiology of pre-eclampsia remains unknown, it appears to have an association with abnormal placentation, involving an imbalance between apoptosis and proliferation within the trophoblast and failure of the spiral arteries to form properly in the myometrium. Such aberrant placentation eventually leads to poor placental perfusion [Brosens, 1977; Pijnenborg et al., 1991; Hemberger, 2007; Roberts and Von Versen-Hoeynck, 2007]. Therefore, placental hypoxia resulting from poor perfusion has been proposed as a mechanism for pre-eclampsia [Jeyabalan et al., 2008].

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During early placentation, hypoxic conditions are required for promotion of trophoblast invasion into the myometrium of the maternal uterus; subsequently, an inadequate oxygen supply induces stress and apoptosis of the trophoblast [Langbein et al., 2008]. Also, hypoxia disturbs normal placentation through induction of apoptosis in cytotrophoblast and syncytiotrophoblast cells, and through stimulation of syncytial knot formation [Jauniaux et al., 2006; Koklanaris et al., 2006]. Results from previous studies have helped to define several clear risk factors for pre-eclampsia; however, controversy over the most effective treatment protocols and diagnostic markers still abounds [Levine et al., 2006; Baumwell and Karumanchi, 2007; Kaaja, 2008]. Therefore, evaluation of existing markers and development of new diagnostic markers for pre-eclampsia is important.

Recently, cDNAs encoding two novel nuclear proteins, the immortalization-upregulated proteins IMUP-1 and IMUP-2, were identified in SV40-immortalized human fibroblast cell lines [Kim et al., 2000]. IMUP-1 and IMUP-2 arise during transcriptional processing through an RNA splicing mechanism [Kim et al., 2003]. IMUPs reportedly influence cellular proliferation, and overexpression of IMUPs is associated with tumorigenesis in various cancer cell lines and tumors [Ryoo et al., 2006]; however, the innate function of IMUPs during human placental development remains unclear. If IMUPs affect cellular proliferation or regulate cellular activities (e.g., differentiation, apoptosis, and invasion) in the trophoblast, obstetrical diseases caused by trophoblast dysfunction, such as pre-eclampsia and intrauterine growth retardation (IUGR), could be a result of inappropriate IMUP expression.

In the present study, we compared expression patterns of IMUP-2 in normal and pre-eclamptic placentas. Furthermore, we examined expression of IMUP-2 in HTR-8/SVneo trophoblast cells exposed to a hypoxic environment, and evaluated the function of IMUP-2 in HTR-8/SVneo trophoblast cells over-expressing IMUP-2.

MATERIALS AND METHODS

PLACENTA SAMPLE COLLECTION

A placenta was considered normal in subjects who underwent a term delivery with no evidence of obstetrical complications ($\geq$37 gestational weeks). Pre-eclampsia was defined as the presence of hypertension (systolic blood pressure of 140 mm Hg 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 obtained from patients in the following groups: (1) term without labor (n = 15); (2) term without labor pre-eclampsia (n = 15); and (3) pre-term without labor pre-eclampsia (n = 11). All women provided written, informed consent prior to collection of samples. Collection of samples and their utilization for research purposes was approved by the Institutional Review Boards of CHA General Hospital, Seoul, Korea. Placental tissues were obtained from the central and peripheral areas of the placentas, snap-frozen in liquid nitrogen, and stored at -80°C, or fixed in DEPC-treated 4% paraformaldehyde (PFA) solution, and embedded in paraffin wax. Alternatively, samples were immediately frozen in OCT compound (SACURA, Tokyo, Japan) for use in RNA in situ hybridization and immunofluorescence.

CELL CULTURE

HTR-8/SVneo trophoblast cells were maintained in RPMI medium 1640 (Gibco; Logan, UT) supplemented with 5% fetal bovine serum (FBS; Gibco) and 100 U/ml penicillin and streptomycin (Gibco). To increase the efficiency of hypoxia treatment, normal medium was exposed to an atmosphere consisting of 99% N_2 and 1% O_2 for about 24 h (Hypoxic medium). Six hours after seeding, the cells were transferred to hypoxic RPMI media and cultured in a hypoxia chamber with reduced atmospheric O_2 (1% O_2, 99% N_2) for 12 and 24 h. At the end of the incubation, cells were collected and cell pellets were kept at -70°C until they were needed for analysis.

TRANSFECTION OF IMUP-2 INTO HTR-8/SVneo TROPHOBLAST CELLS

For transfection, 1×10^6 HTR-8/SVneo trophoblast cells were seeded onto 100×20-mm culture dishes. According to the manufacturer's instructions, when cells reached 80% confluence, they were transfected with 8 μg of IMUP-2 expressing plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Transfected cells were maintained in normal media (5% FBS) for 24 h and lysed in lysis buffer (Intron, Seoul, Korea).

SEMI-QUANTITATIVE RT-PCR (QRT-PCR)

Total RNA from frozen placental tissues and HTR-8/SVneo trophoblast cells were extracted using TRIzol (Invitrogen). A reverse transcription reaction was performed with 1 μg purified total RNA using Superscript III reverse transcriptase (Invitrogen). Synthesized cDNA was amplified by PCR. Primers used for RT-PCR analysis are presented in Table I. PCR products were visualized by electrophoresis on a 1.2% (w/v) agarose gel (Cambrex, Rockland, ME) containing 0.5 μg/ml ethidium bromide (Promega, Madison, WI), and were visualized using a video image analyzer (Bio-Rad,

TABLE I. Primers Sequences for Semi-Quantitative Reverse Transcriptase-Polymerase Chain Reaction Used in This Study

Name	Sequence (5′–3′)
IMUP-1	ATGGAGTTCGACCTGGG
	TCAGTGGGGAGCCTCCTT
IMUP-2	ATGGAGTTCGACCTGGG
	ACTTCACATCCGTGTCCG
XIAP	GTGACTAGATGTCCACAAGG
	CTTGAGGAGTGTCTGGTAAG
BIR-1	GGAATTCATGACTTTTAACAGTTTTGAAGG
	CTTGAAGCTTGTCCTCAGGATCCCAGATAGTTTTCAAG
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
28srRNA	TTGAAAATCCGGGGGAGAG
	ACATTGTTCCAACATGCCAG

Hercules, CA). Optical densities of the genes of interest and the 28s rRNA bands were quantified by densitometry using the Quantity One program.

RNA IN SITU HYBRIDIZATION

An in situ hybridization detection kit (InnoGenenx, San Ramon, CA) was used for detection of IMUP-2 mRNA expression in placental tissues. Briefly, the probe was prepared using two primers: IMUP2/F (5′-ATGGAGTT CGACCTGGG-3′) or IMUP2/R (5′-ACTTCACAT-CCGTGTCCG-3′). Riboprobes were synthesized from linearized plasmids containing the indicated cDNA using DIG-labeled UTP (Roche, Mannheim, Germany) and in vitro transcription kits (Promega). IMUP2 was transcribed using an SP6 RNA polymerase for generation of the anti-sense probe and a T7 RNA polymerase for generation of the sense probe (as a negative control). Placental tissues were treated with proteinase K solution (Dako, Glostrup, Denmark) for 10 min at 37°C and post-fixed with 1% formaldehyde solution. DIG-labeled IMUP-2 probes were applied to the placental tissues, denatured in a heat block at 85°C for 5 min, and incubated overnight in a 37°C humidified chamber. Following hybridization, biotin/streptavidin-ALP immunodetection-based methods were used for tissue detection.

WESTERN BLOT ANALYSIS

Placental tissues and cell lysates were homogenized with extraction solution (Intron, Kyunggi, South Korea) and centrifuged at 12,000 rpm for 15 min at 4°C. For analysis of sub-cellular localization of IMUP-2 in 1% hypoxia-treated cells and IMUP-2-transfected cells, nuclear and cytoplasmic fractions were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce, Rockford, IL), according to the manufacturer's instructions. Total protein extracts (20–40 μg) were heated at 95°C for 5 min, resolved by 12% SDS-Polyacrylamide gel electrophoresis (PAGE), and electrotransferred onto PVDF membranes at 100 V for 80 min. The membranes were blocked by TBS-T with 5% nonfat milk, and then incubated overnight at 4°C with anti-IMUP 2 (1:1,000; AbFRONTIER, Seoul, Korea), anti-caspase-9 (1:4,000; R&D systems), anti-Bcl-2 (1:500; Santa Cruz Biotechnology), anti-Bak (1:2,000; EPITOMICS), or anti-β-actin (1:5,000; Santa Cruz Biotechnology) antibodies, followed by horseradish peroxidase (HRP)-conjugated rabbit (1:5,000) or mouse (1:10,000) secondary antibodies (Bio-Rad). Peroxidase activity was visualized using an ECL Advance Western blotting detection kit (Amersham, Piscataway, NJ).

IMMUNOSTAINING

Using a graded ethanol series, paraffin-embedded tissue was dewaxed and rehydrated. Sections were immersed in a proteinase K solution (Dako) for 5 min; for blocking of nonspecific binding, the slides were then incubated for 30 min at room temperature in blocking solution (Dako), followed by overnight incubation with anti-IMUP-2 (1:200; AbFRONTIER) at 4°C 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. Following incubation with streptavidin-HRP (Dako), immunoreactive proteins were detected using 3,3-diaminobenzidine tetrahydrochloride (DAB) substrate-chromogen solution (VECTOR, Burlingame, CA). Slides were counterstained with Mayer's hematoxylin (Dako) and mounted. For immunofluorescence, frozen sections and HTR-8/SVneo trophoblast cells exposed to hypoxic conditions were fixed with cold methanol. Sections and cells were blocked with normal blocking serum (Dako) for 30 min and incubated overnight at 4°C with anti-M30 (1:50; Roche) and anti-IMUP-2 (1:50; AbFRONTIER), and, subsequently with 1:200 Alexa Fluor 488 chicken anti-mouse IgG (1:200; Molecular Probes, Inc.) or Rhodamine-conjugated chicken anti-rabbit secondary antibody (1:400; Molecular Probes, Inc.) for 1 h. Stained slides were mounted with a glycerol-based mounting solution containing 1, 4-diazabicyclo-octane with propidium iodide (Sigma, St. Louis, MO). Additionally, PI staining was used for detection of nuclear segmentation of trophoblasts exposed to hypoxic conditions caused by apoptosis. All images were acquired using an LSM 510 META confocal microscope (Carl Zeiss, Inc., Switzerland).

STATISTICS

Data represent the mean ± SD, and were analyzed for statistical significance ($P < 0.05$) using Student's *t*-test. All experiments were performed in duplicate and were repeated at least three times.

RESULTS

To confirm expression of IMUPs in placental tissues, we conducted semi-quantitative RT-PCR analysis of IMUP-1 (357 bp) and IMUP-2 (261 bp) expression in various placental tissues, and compared gene intensity to 28s rRNA (100 bp) expression. We observed no statistically significant differences in IMUP-1 expression among normal, second-trimester pre-eclamptic, and third-trimester pre-eclamptic placentas (Fig. 1A); however, compared to normal and third-trimester pre-eclamptic placentas, expression of IMUP-2 was significantly elevated in second-trimester pre-eclamptic placentas (Fig. 1B, $P < 0.001$). We used the Western blot assay for analysis of quantitative expression of IMUP-1 and IMUP-2 in placental tissues. Despite the fact that when compared with normal placentas, expression of IMUP-1 shows a tendency to increase only in the central portion of second-trimester pre-eclamptic placentas, there are no differences in the peripheral portion of the placenta between normal and pre-eclamptic placentas. Otherwise, results from Western blot analysis showed that quantitative expression of IMUP-2 increased more in the peripheral portion of pre-eclamptic placental tissues than in normal placental tissues (Fig. 1C). Also, compared with normal placental tissues, increased HIF-1a expression was observed in the peripheral portion of pre-eclamptic placental tissues (Fig. 1C). According to these results, IMUP-1 and IMUP-2 are generated through alternative splicing from the same primary transcript; however, their expression could be controlled by different mechanisms, such as post-transcriptional modification. Also, increased IMUP-2 expression could be associated with pre-eclampsia. In addition, expression of IMUP-2 was more enhanced in peripheral regions than in central regions of pre-eclamptic

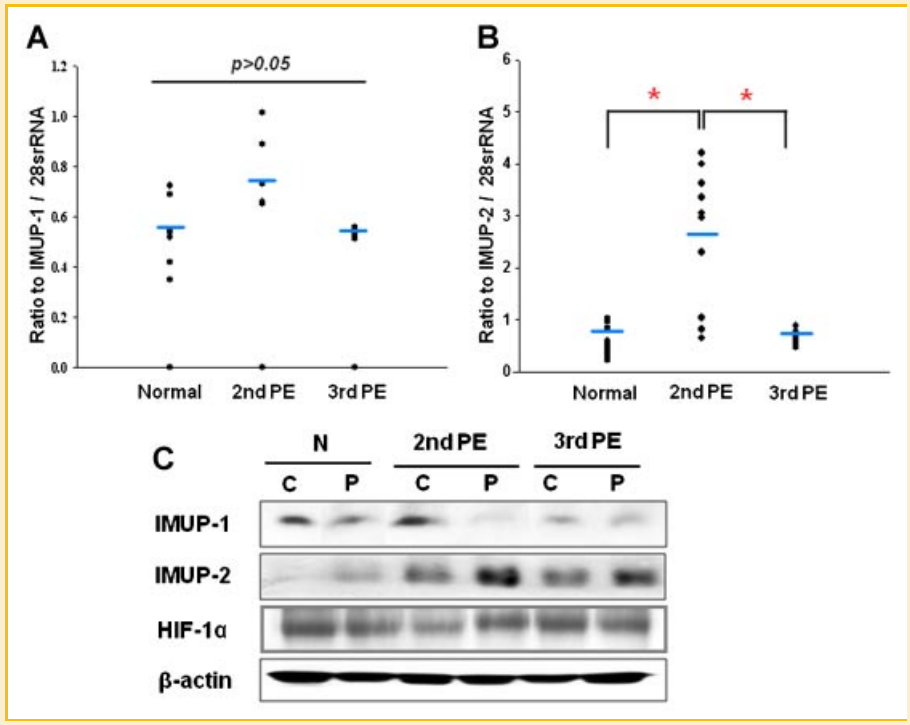

Fig. 1. Expressions of IMUP-1 and IMUP-2 in normal and pre-eclampsia placentas. A: Densitometric analysis of IMUP-1 mRNA expression using semi-quantitative RT-PCR. B: Densitometric analysis of IMUP-2 mRNA expression relative to 28s rRNA mRNA expression. C: Expression of IMUP-1 and IMUP-2 in normal and pre-eclampsia placentas using Western blot analysis. β-Actin was used as an internal control. PE, pre-eclampsia; C, center portion; P, peripheral portion. Data are expressed as means ± SD. Statistical analysis was carried out by unpaired Student's *t*-test ($^*P < 0.001$).

placentas. These results suggest the possibility that different IMUP-2 levels in the central and peripheral regions of the placenta could reflect a lower oxygen content in the peripheral region under hypoxic conditions.

For localization of IMUP-2 within the placenta, we performed RNA in situ hybridization using a pcDNA3 plasmid containing IMUP-2 cDNA. We constructed a probe with DIG-conjugated IMUP-2 using in vitro nick translation. In normal placentas, we observed

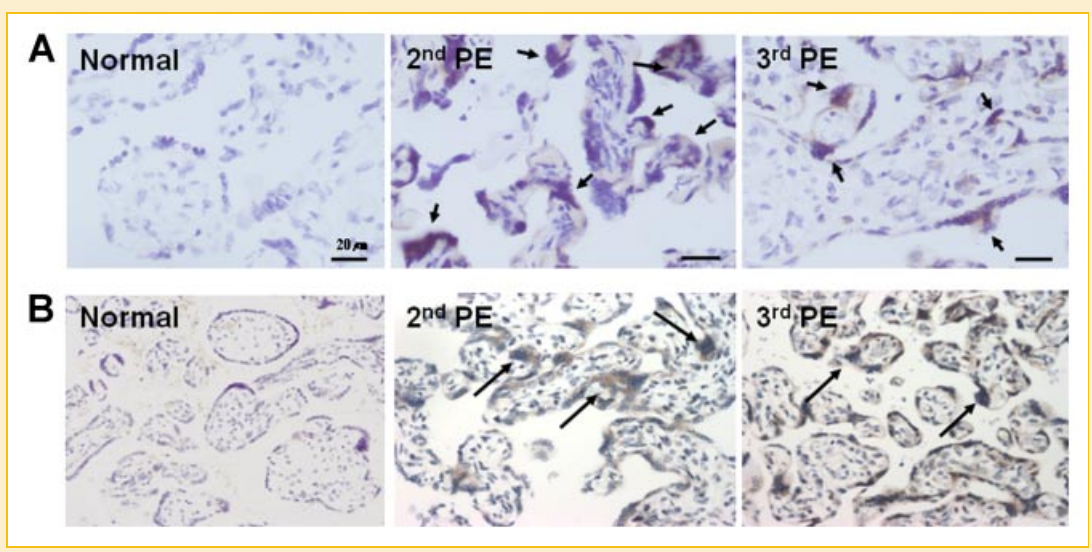

Fig. 2. Expression of IMUP-2 in normal and pre-eclampsia placentas using histological analysis. A: Expression of IMUP-2 in placental tissues using mRNA in situ hybridization (100×). Arrow means positive cells for IMUP-2 mRNA expression. B: Immunohistochemistry analysis using IMUP-2 antibody in placental tissues. Arrow means positive cells for IMUP-2 in syncytiotrophoblasts and syncytial knots of placenta tissue (200×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

IMUP-2 mRNA expression in syncytiotrophoblasts, syncytial knots, and decidual cells of the placental villi and the placental basal plate. Interestingly, IMUP-2 mRNA expression was strong in syncytiotrophoblasts and syncytial knots within second-trimester pre-eclamptic and third-trimester pre-eclamptic placentas (Fig. 2A). To confirm whether or not IMUP-2 expression in syncytiotrophoblasts and syncytial knots of the placenta correlated with abnormal placentation, we assessed IMUP-2 expression using immunohistochemistry. In accordance with results from RNA in situ hybridization, IMUP-2 expression in syncytiotrophoblasts and syncytial knots from second-trimester pre-eclamptic placentas was significantly higher than expression in normal placentas (Fig. 2B). These expression patterns for IMUP-2 were similar in third-trimester pre-eclamptic placental tissue. To determine whether or not pre-eclampsia correlated with trophoblastic apoptosis, we analyzed expression of M30 (an apoptosis-specific marker for trophoblasts) and caspase-3 in placentas. As shown in Figure 3, expression of M30 was weakly expressed in syncytiotrophoblasts and syncytial knots in normal placental tissues; otherwise, M30 expression increased substantially in syncytiotrophoblasts and syncytial knots of pre-eclamptic placentas (Fig. 3A). In results from Western blot analysis, expression of caspase-3 showed a greater increase in pre-eclamptic placentas than in normal placentas, particularly, in second-trimester pre-eclamptic placentas (Fig. 3B). These findings indicate that apoptosis increased in pre-eclampsia and increased M30-positivity in syncytiotrophoblasts and syncytial knots involved in progress of apoptosis of trophoblasts, thus promoting the abnormal placentation associated with pre-eclampsia. Therefore, increased expression of IMUP-2 in syncytiotrophoblasts and syncytial knots may be associated with apoptosis of the trophoblast.

To explore the question of whether or not expression of IMUP-2 correlated with trophoblastic apoptosis under hypoxic conditions, we assessed IMUP-2 expression in hypoxia-exposed HTR-8/SVneo cells using RT-PCR and Western blot. IMUP-2 mRNA expression was higher in hypoxia-exposed HTR-8/SVneo cells than in cells grown under normoxia conditions; otherwise, there was no difference in IMUP-1 mRNA expression, regardless of hypoxia (Fig. 4A). Western blot analysis confirmed increased expression of HIF-1α under hypoxic conditions. Similar to mRNA expression patterns, there was no difference in expression of IMUP-1 in HTR-8/SVneo cells according to hypoxic treatment. However, expression of IMUP-2

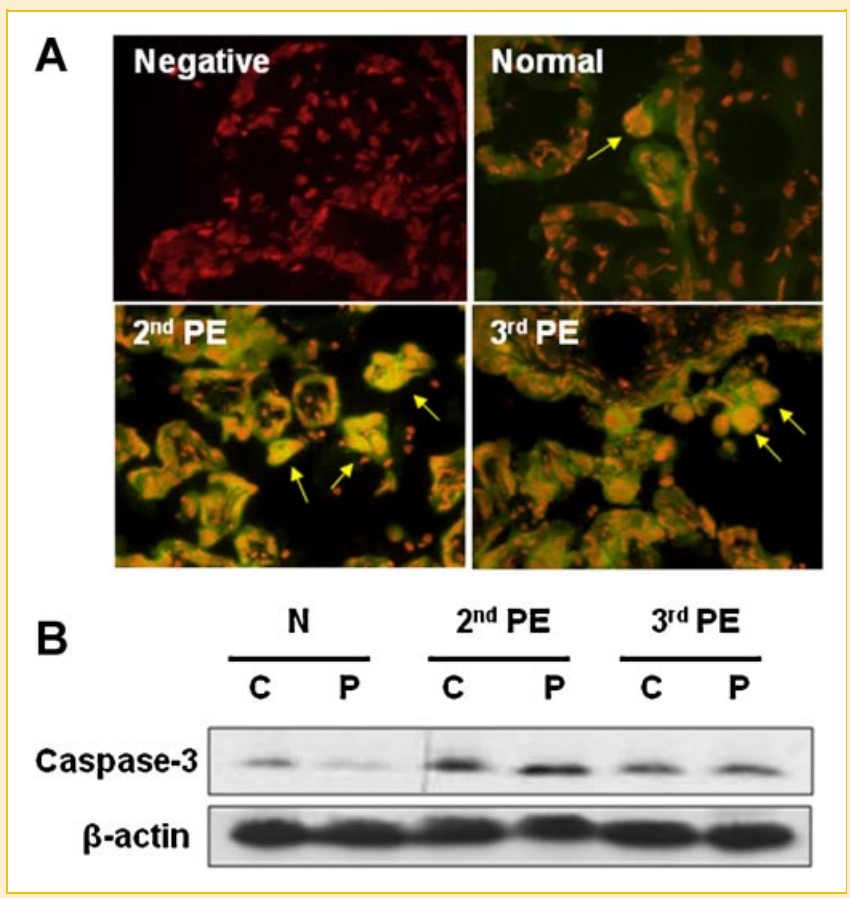

Fig. 3. Apoptosis in normal and pre-eclampsia placentas. A: Expression of M30 in normal and pre-eclampsia placentas using immunofluorscence. Yellow arrow means positive cells for M30, a specific marker for apoptosis of trophoblast, in syncytiotrophoblasts and syncytial knots of placenta tissue (200×). B: Expression of caspase-3 in normal and pre-eclampsia placentas using Western blot analysis. PE, pre-eclampsia; C, center portion; P, peripheral portion. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

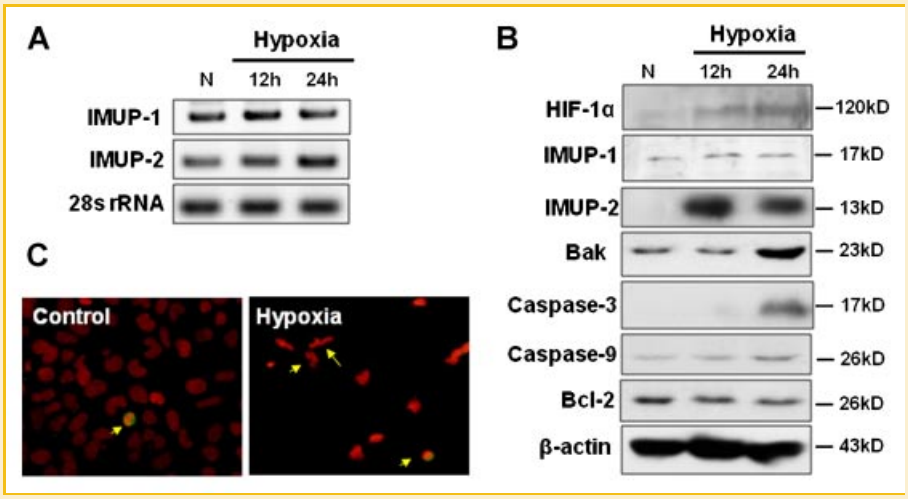

Fig. 4. Expressions of IMUP-1 and IMUP-2 in HTR-8 SV/neo trophoblast cell line under hypoxic conditions. A: mRNA expressions of IMUP-1, IMUP-2, and 28s rRNA during hypoxia. B: Expressions of HIF-1a, IMUPs, and apoptosis-related proteins in HTR-8 SV/neo trophoblast cell line under hypoxic conditions using Western blot analysis. C: Apoptosis analysis in HTR-8 SV/neo trophoblast cell line using PI staining during hypoxia. β-Actin was used as an internal control. Arrow means nuclear abnormalities in HTR-8SV/neo trophoblast cells exposed to hypoxic condition (200×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

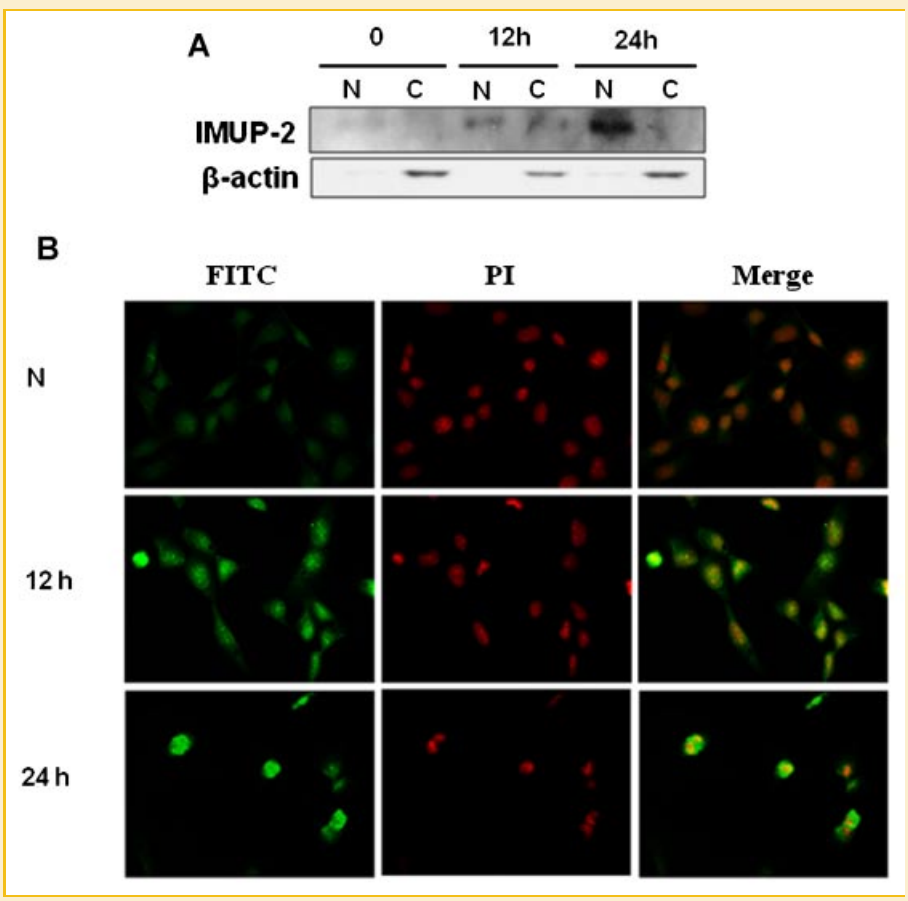

Fig. 5. Localization of IMUP-2 in HTR-8 SV/neo trophoblast cell line under hypoxic conditions. A: IMUP-2 expression was increased in the nuclear fraction of HTR-8 SV/neo trophoblast cell line under hypoxic condition. B: Immunofluorescence for IMUP-2 in HTR-8/SVneo trophoblast cells exposed to hypoxia. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

and other apoptosis-related factors, such as BAK, Caspase-3, and Caspase-9, increased under hypoxic conditions; however, expression of anti-apoptosis factor Bcl-2 decreased (Fig. 4B). Under hypoxic conditions, abnormal phenotypes, including nuclear fragmentation, were observed in the morphologies of HTR-8/SVneo trophoblast cells (Fig. 4C). These results indicate that increased IMUP-2 expression in HTR-8/SVneo trophoblast cells exposed to hypoxic conditions could be induced abnormal phenotypes of the nucleus and expression of apoptosis-related proteins, followed by apoptosis of trophoblasts.

To examine localization and expression of IMUP-2 under hypoxic conditions, we performed subcellular fractionation. Under normoxic conditions, IMUP-2 was weakly detected in the nucleus. Expression of IMUP-2 increased when the cells were exposed to hypoxic conditions for 12 and 24 h (Fig. 5A). These results were confirmed by immunocytochemistry (Fig. 5B), suggesting that IMUP-2 expression in HTR-8/SVneo trophoblast cells increases under hypoxic conditions. In addition, increased expression of IMUP-2 correlated with apoptosis of trophoblasts during hypoxia.

To investigate the question of whether or not ectopic IMUP-2 expression triggered trophoblastic apoptosis, we transfected HTR-8/SVneo trophoblasts with IMUP-2 for 6, 12, and 24 h, and analyzed apoptosis; specifically, we counted the total number of surviving cells and analyzed expression of apoptosis-related genes. As shown in Figure 6, IMUP-2 transfection altered the morphology of HTR-8/SVneo trophoblast cells and decreased cell numbers by approximately 30% when compared with mock-transfected controls (Fig. 6A,B, $P < 0.05$). Moreover, overexpression of IMUP-2 in HTR-8/SVneo trophoblast cells after 24 h of treatment significantly induced expression of pro-apoptotic genes, such as caspase-3, caspase-9, and Bak when compared with mock transfection (Fig. 7A, $P < 0.05$). Also, expression of the anti-apoptotic marker Bcl-2 was reduced when compared with mock-transfected cells (Fig. 7A, $P < 0.001$). Results from Western blot analysis showed that pro-apoptosis-related protein expression and Bcl-2 as an anti-apoptosis-related protein were increased and decreased in a time-course dependent manner, respectively (Fig. 7B). These expression patterns were similar to results from RT-PCR. These results suggested that overexpression of IMUP-2 by hypoxia could be induced apoptosis in HTR-8/SVneo trophoblast cells.

DISCUSSION

The trophoblast, particularly the syncytiotrophoblast, is a continuous, uninterrupted layer that extends over the surfaces of all villous

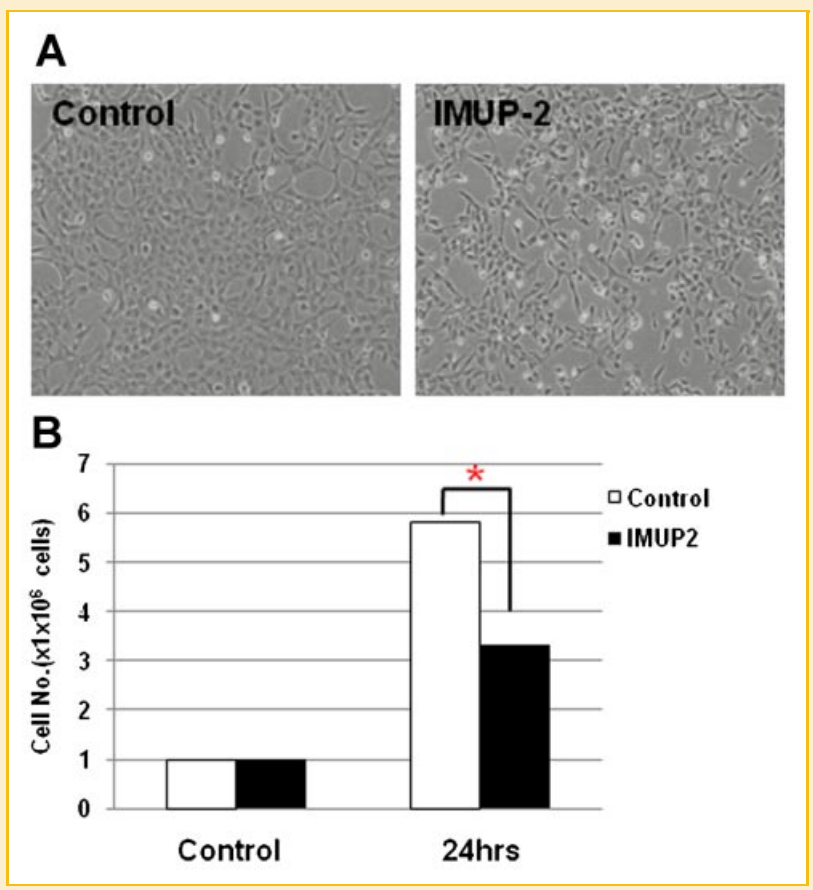

Fig. 6. Apoptosis in IMUP-2-transfected HTR-8/SVneo trophoblast cell line. A: The morphology of control cells and over-expressing IMUP-2 (100×). B: Cell growth differences between control and IMUP-2 over-expressing HTR-8/SVneo trophoblast cell line. Statistical analysis was carried out using unpaired Student's *t*-test (*$P < 0.05$).

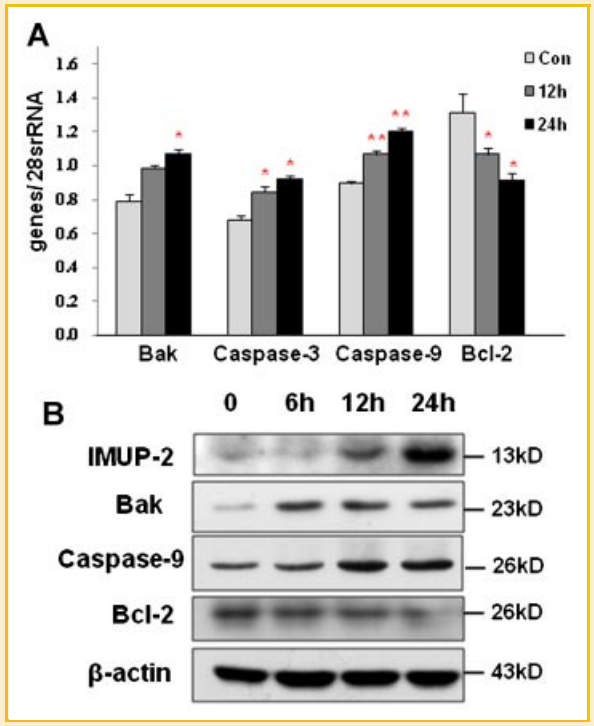

Fig. 7. Expression of IMUP-2 in IMUP-2-transfected HTR-8/SVneo trophoblast cell line. A: mRNA expression and densitometric analysis of apoptosis-related genes in IMUP-2 transfected HTR-8/SVneo trophoblast cell line, relative to 28s rRNA. B: Western blot analysis of apoptosis-related genes. Statistical analysis was carried out using unpaired Student's *t*-test ($^{*}P < 0.05$,

trees and over parts of the inner surfaces of the chorionic and basal plates during placental development. Survival of the syncytiotrophoblast depends on its fusion with cytotrophoblasts, and the associated apoptotic mechanism is a kind of cellular death that involves formation of syncytial knots. These structures are heterogeneous, specialized, and characterized by accumulation of syncytial nuclei [Huppertz et al., 2005; Langbein et al., 2008]. In placental development, the number of syncytiotrophoblasts and syncytial knots most clearly represents trophoblastic apoptosis through cell-to-cell fusion; in certain pathological instances, they also exhibit excessive trophoblastic apoptosis during placental development, which leads to placental diseases, such as pre-eclampsia and intrauterine growth restriction (IUGR), and can ultimately result in fetal death. Especially, aberrantly-upregulated apoptosis in first-trimester extracellular trophoblasts and hypoxia-induced syncytial knotting correlate with progression of severe pre-eclampsia [Ishioka et al., 2006; Heazell et al., 2007; Whitley et al., 2007]. Thus, enhanced apoptosis of the syncytiotrophoblast could induce severe pre-eclampsia through reduction of the number of trophoblasts in the placenta. Conversely, there is evidence that an excess of proliferative immature intermediate trophoblasts also leads to pre-eclampsia and IUGR [Redline and Patterson, 1995; Jeschke et al., 2006]. Therefore, it appears that a delicate balance between proliferation and apoptosis of trophoblasts, including cytotrophoblasts and syncytiotrophoblasts, may play a significant role in maintenance of a healthy placenta [Huppertz et al., 2006].

Hypoxic conditions are necessary for promotion of trophoblast invasion into the myometrium of the maternal uterus during early pregnancy; subsequently, an inadequate oxygen supply induces stress and apoptosis of the trophoblast, which can lead to pre-eclampsia [Lash et al., 2007; Huppertz et al., 2009]. Hypoxia-inducible factor (HIF-1) induced by hypoxic conditions is a transcriptional activator that regulates expression of several genes in response to oxygen tension [Dunwoodie, 2009]. In particular, HIF-1a expression is regulated by lower O_2 levels, which are elevated during the early stage of the first trimester of gestation, and decrease with gestational age [Wang et al., 1995]. This invasion of adequate trophoblasts and defective spiral arteries that are transformed during the first trimester may result in impaired placental perfusion and chronic placental ischemia and hypoxia after gestation, leading to adverse pregnancy outcomes [Rajakumar and Conrad, 2000]. Therefore, it is likely that consistent increased expression of HIF-1a under hypoxic conditions may play a role in change of cellular phenotypes and cellular functions through stimulation of several genes during placental development.

In the present study, we describe a novel function of the immortalization-upregulated protein IMUP-2 in placental development. Expression of IMUP-1 and IMUP-2 correlates with cellular proliferation and oncogenesis in various cancer cell lines [Kim et al., 2000; Ryoo et al., 2006]. In in vitro experiments, overexpression of IMUPs has resulted in mediated tumorigenesis, increased levels of S and G2/M phase cells, and induction of p53 pathway-related mutations; however, the precise role of IMUPs in normal biological activities remains a mystery. Here, we investigated the question of whether or not alterations in IMUP expression in the placenta correlated with pathological consequences.

Expression of IMUP-1 remained the same in normal and pre-eclamptic placentas (Fig. 1A). However, expression of IMUP-2 in proliferating syncytiotrophoblasts and syncytial knots of the placental villi was significantly upregulated in severe second-trimester pre-eclampsia when compared with normal and third-trimester pre-eclampsia. Interestingly, results from Western blot analysis showed that expression of IMUP-2 was more enhanced in peripheral regions than in central regions of pre-eclamptic placentas. It is likely that different IMUP-2 levels in the central and peripheral regions of the pre-eclamptic placenta could reflect a lower oxygen content in the peripheral region under hypoxic conditions. Additionally no difference was observed in IMUP-1 expression in HTR-8/SVneo trophoblast cells under hypoxic conditions (Fig. 4A,B). Otherwise, IMUP-2 was upregulated in HTR-8/SVneo trophoblast cells under hypoxic conditions, and upregulated IMUP-2 caused apoptosis of trophoblasts by induction of caspase-3 expression and suppression of Bcl-2 expression. Although results from Western blot analysis showed that IMUP-2 mRNA expression was significantly enhanced in severe second-trimester pre-eclampsia, IMUP-2 expression in third-trimester pre-eclampsia was also higher than in the normal control. These findings indicate different functions between IMUP-1 and IMUP-2 during placental development. In addition, these results suggest that IMUP-2 is associated not only with cellular proliferation but also with trophoblastic apoptosis, particularly in placental disorders, such as severe pre-eclampsia. It is not clear which mechanism plays

the dominant role in mediation of IMUP-2 expression in placental development. Subsequent IMUP-2 studies in the developing placenta might provide a more detailed correlation between expression of IMUP-2 and the function of the trophoblast, particularly with regard to cell fusion mechanisms and invasion activity. Furthermore, our data suggest that IMUP-2 may be a viable diagnostic marker for pre-eclampsia. Currently, there are few useful markers for diagnosis of pre-eclampsia. Our results support the idea that pre-eclampsia results from aberrant apoptosis and dysfunction of syncytiotrophoblasts, and our data provide a better understanding of the function of IMUP-2 in the developing placenta.

In conclusion, IMUP-2 expression is specifically elevated in preterm pre-eclampsia and under hypoxic conditions, and IMUP-2 induces apoptosis of the trophoblast; therefore, IMUP-2 might have functional involvement in placental development and in gynecological diseases, such as pre-eclampsia. Moreover, we reveal that IMUP-2 might fit the criteria for a new candidate marker for early diagnosis of pre-eclampsia.

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