Placental HtrA3 Is Regulated by Oxygen Tension and Serum Levels Are Altered during Early Pregnancy in Women Destined to Develop Preeclampsia

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Context: The pathogenic origin of preeclampsia is defective placental development (placentation) and function. Preeclampsia is not diagnosed until later in pregnancy, and reliable early detection is highly desirable. HtrA3 is a recently cloned gene with high expression during placentation in the mouse, rhesus monkey, and human.

Objective: The present study examined the placental production and the serum profile of HtrA3 across gestation in women, the potential molecular mechanisms regulating HtrA3 production, and the association between maternal HtrA3 serum levels and preeclampsia.

Methods: Immunohistochemistry determined HtrA3 expression pattern and cellular localization in first-, second-, and third-trimester placenta. The maternal serum HtrA3 levels were analyzed by Western blotting. The regulation of placental HtrA3 production and the secretion by oxygen tension was investigated in first-trimester placental explants and trophoblast cells.

Results: Placental HtrA3 protein was maximally produced in the first trimester and then dramatically down-regulated, especially in the syncytiotrophoblast. HtrA3 was secreted into the maternal circulation with a serum profile reflecting placental production. Oxygen tension regulated HtrA3; low oxygen enhanced, whereas the transition from low to high oxygen decreased, HtrA3 protein production in syncytiotrophoblast. Maternal serum HtrA3 levels at approximately 13–14 wk of gestation were significantly higher in women who subsequently developed preeclampsia. It appeared that HtrA3 down-regulation was delayed in preeclamptic pregnancies.

Conclusions: HtrA3 protein production is closely associated with changing in oxygen tension in the placenta. The decline in HtrA3 at the end of first trimester may reflect the placental low to high oxygen switch. Abnormally high levels of serum HtrA3 at approximately 13–14 wk of gestation is associated with preeclampsia. (J Clin Endocrinol Metab 96: 403–411, 2011)

Preeclampsia is a serious disorder of human pregnancy affecting 5–10% of pregnant women (1). It contributes significantly to both maternal and fetal morbidity and mortality, accounting for about 18% of maternal and perinatal deaths in industrialized countries (2, 3). Preeclampsia is not diagnosed until it becomes clinically apparent late in pregnancy, at which time delivery is the only effective cure, often inflicting prematurity. The burden of preeclampsia thus also falls on the neonate because fetal survival rates inversely correlate with gestational age; survival rate for a baby born at 23 wk is less than 10% (4).
Although the pathology of preeclampsia is established early in gestation, there is no reliable test that accurately predicts the development of the disease with sufficient sensitivity and specificity to be clinically useful (5). The exact cause of preeclampsia is not completely clear, although it is widely accepted that preeclampsia requires the presence of a placenta and that faulty development and/or function of the placenta is the most likely cause (6, 7). It is suggested to be a two-stage disorder (8): first stage, abnormal placental development in early pregnancy likely resulting in abnormal release of placent al factors into the maternal circulation; and second stage, the maternal systemic pathophysiological responses to the placental aberrations leading to the clinical manifestations of preeclampsia (8).

One critical event of normal placental development (placentation) is the widening (remodeling) of the spiral arteries of the mother’s uterus that deliver blood into the placenta. This process depends on a specialized placental cell type, extravillous cytotrophoblast (EVT), which is invasive in nature. EVTs migrate into the spiral arteries and replace the endothelial lining, and convert the narrow and high-resistance spiral arteries into widened and low-resistance channels (6), allowing expanded capacity of the uteroplacental circulation to support the growing fetus (9). In contrast, in preeclamptic placentas, the spiral arteries are only partially remodeled such that blood flow into the placenta is lower than in normal pregnancy (6, 7, 9).

During the initial stages of placentalation (before 10–12 wk of gestation), when the EVTs migrate into the spiral arteries, they form plugs and block the arteries, preventing blood flow to the placenta and creating a low-oxygen environment required for fetal organogenesis and the development of other placental cell types (9, 10). At around 13–14 wk of gestation, the spiral arteries start to be de-plugged and remodeled, resulting in a dramatic increase in blood flow and oxygen concentration (10). This oxygen switch is a critical milestone in placentation. Impaired dissolution of the plugs could be a cause of defective vessel remodeling in preeclamptic placentas (9). Accordingly, the oxygen switch would be blunted and/or delayed, causing prolonged placental hypoxia in pregnancies destined to develop preeclampsia. Identifying abnormalities in the oxygen switch (indicating vessel remodeling abnormalities) at early stages would likely identify pregnancies that are at risk for developing preeclampsia.

We previously identified and cloned HtrA3, a serine protease that is dramatically up-regulated during placentalation in the mouse, rhesus monkey, and human (11–15). Four mammalian HtrA family proteins (HtrA1, -2, -3, -4) have been identified and have roles in apoptosis, arthritis, neurodegenerative and neuromuscular disorders, age-related macular degeneration, and cancer (16–20).

In the mouse, HtrA3 mRNA expression was highest in the developing placenta (12), with HtrA3 protein being produced in maternal decidual cells anchoring the early placenta and in the fetal capillary endothelium (13). In human first-trimester placenta, HtrA3 is similarly highly produced in maternal decidual cells and in certain trophoblast cell types (13). Placental HtrA3 is secreted into the maternal circulation and clearly detectable in serum of pregnant women in the first trimester (13).

In this study, we first examined the pattern of placental production and serum profile of HtrA3 across gestation. Placental HtrA3 protein was maximally produced in the first trimester and then dramatically down-regulated. This dynamic pattern was reflected in maternal serum HtrA3. The down-regulation coincided temporally with the placental oxygen low-to-high concentration switch (~13–14 wk). Serum HtrA3 levels at around this time (~13–14 wk) were significantly altered in women who subsequently developed preeclampsia compared with normal pregnancies. Using placental explant and cell culture models, we demonstrated that oxygen tension regulated placental HtrA3 production, providing a clear molecular basis for HtrA3 protein regulation during normal placentation and for why abnormal serum HtrA3 levels around 13–14 wk of pregnancy may indicate abnormalities in the placental oxygen switch and hence the risks of developing preeclampsia.

Materials and Methods

Placental collection and processing

Placenta from the first (8–12 wk gestation, n = 10) and second (14–19 wk, n = 3) trimester and first-trimester decidua (n = 7) were obtained during elective termination of pregnancy. Term placenta (third trimester, 37–39 wk gestation, n = 8) were from healthy women with singleton pregnancies undergoing elective cesarean section. All collections were under ethics approval (Southern Health Human Research Ethics Committee) with written informed consent. Tissues were formalin fixed.

Immunohistochemistry

Five-micrometer sections were subjected to immunohistochemistry (13) using a primary sheep anti-HtrA3 antibody or preimmune sheep IgG (negative control). Trophoblast cells were identified by immunostaining for cytokeratin [antihuman cytokeratin monoclonal antibody (7 μg/ml; BD Biosciences, San Jose, CA; on serial sections)]. The relative intensity of the immunostaining in different placental cell types in the first, second, and third trimesters of pregnancy was scored semiquantitatively by two independent observers (0, no stain, to 4, maximal stain) and expressed as the mean intensity (±SEM).

Maternal serum collection

Maternal serum was collected from healthy women (a different cohort from the placental collection) at various stages of singleton pregnancy in the first (8–12 wk gestation, n = 5) and...
third (term) trimesters (n = 5). Sera from 10–12 (n = 7/group), 13–14 (n = 8/group), and 37–39 (n = 6/group) wk of gestation in women who subsequently developed preeclampsia, and matching normal controls (matched for maternal age, collection time, gestation at blood collection, parity, smoking status) were retrieved from a blood bank collected between 1999 and 2001 (n ≥ 7 per group). Preeclampsia diagnosis was based on blood pressure elevation (systolic ≥140 or diastolic ≥90 mm Hg) and new-onset proteinuria (≥2+ on dipstick or urinary protein to creatinine ratio >0.3). The subjects were normotensive during early pregnancy or postpartum without a history of chronic hypertension.

**Detection of HtrA3 protein in serum**

HtrA3 levels in maternal sera were determined by Western blotting (13) after the removal of major subclasses of human γ-globulin (IgG) by incubating 10 μl serum with 20 μl protein G agarose (Roche, Castle Hill, NSW, Australia). All samples for comparison were loaded on the same gel for Western analysis. Relative protein level was determined densitometrically based on equal protein loading.

**Placental explant cultures**

First-trimester placental (8–10 wk) explants were established from terminal regions of chorionic villi (21). Villous tips (35–45 mg/well) were equilibrated in DMEM/F12 at 20% O2 for 48 h. After medium change, half were cultured at 20% O2 and the other half at 3% O2 with conditioned media harvested at 24 h. Culture media from villi derived from the same placenta were subjected to Western analysis as above.

**BeWo cell culture and analysis**

BeWo cells (American Type Culture Collection, Manassas, VA), at 70% confluence, were syncytialized using 20 μM forskolin or vehicle (dimethyl sulfoxide DMSO) for 48 h. Syncytialization was confirmed by significant down-regulation of E-cadherin [Western analysis with anti-E-cadherin (Invitrogen, Carlsbad, CA)], and approximately 7-fold increased β-human chorionic gonadotropin (hCG) in conditioned media (Pathology Services, Southern Health, Melbourne, Australia). Nonsyncytial or syncytial BeWo cells were cultured in fresh serum-free media for 24 h under 2.5 or 20% O2 conditions. To examine the effect of oxygen tension transition from 2.5 to 20%, cells were cultured at 20% O2 and the other half at 3% O2 with conditioned media harvested at 24 h. Culture media from villi derived from the same placenta were subjected to Western analysis above.

RNA was isolated from BeWo cells using an RNasy mini-kit (QIAGEN GmbH, Hilden, Germany), DNA-free RNA (2 μg) reverse transcribed using SuperScript III reverse transcriptase (Invitrogen) with random primers, and quantitative real-time RT-PCR performed using a Roche LightCycler (Roche, Castle Hill, New South Wales, Australia). The primers were: 5’-AGGGGCTGTCACATGAAGA-3’ and 5’-GCTCCGCTAATTTCCAGT-3’ (for HtrA3) and 5’-CGCTACCACATC-CAAGGAA-3’ and 5’-GTGGATTACCGCGGCT-3’ (for 18S). The ratio HtrA3 to 18S was calculated and relative HtrA3 expression expressed as percent control.

**Statistical analysis**

Data are expressed as mean ± SEM. All culture experiments were repeated independently at least three times. Comparisons of HtrA3 immunostaining intensities used one-way ANOVA and Tukey’s test [PRISM version 5 for Windows (GraphPad Software Inc., San Diego, CA)]. Comparisons between serum samples used the Mann-Whitney test. Other comparisons between two parameters or two groups used paired Student t test. P < 0.05 (two tailed) was considered significant.

**Results**

**Placental HtrA3 protein is maximal in the first trimester and then down-regulated**

The expression pattern and cellular localization of HtrA3 in the human placenta across gestation were determined by immunohistochemistry. Overall levels were highest in first-trimester placenta (8–12 wk gestation, Fig. 1) when strong immunoreactive HtrA3 was present in floating villi, anchoring villi, trophoblast shell, and endovascular EVT (Fig. 1, A and B). Immunostaining for cytokeratin on serial sections confirmed trophoblast identity. In the floating villi, staining was more intense in syncytiotrophoblast than in cytotrophoblast or villous stroma (Fig. 1A). Anchoring villi showed a clear gradient of HtrA3 staining, with no expression proximally but strong staining distally (Fig. 1A). The trophoblast shell, the continuum of the distal edge of the anchoring villi, was also strongly positive for HtrA3. The endovascular EVTs, trophoblast shell derivatives that have invaded the maternal spiral arteries, were also highly positive for HtrA3 (Fig. 1A). In contrast, interstitial EVTs (also of trophoblast shell origin but invading the maternal decidua) were, although positive for cytokeratin, negative for HtrA3 (Fig. 1A). In the first trimester, strong HtrA3 protein was also detected in stromal decidual cells and endometrial glandular epithelia (Fig. 1B).

The overall placental HtrA3 was down-regulated after the first trimester (Fig. 1B). In the second and third trimesters, floating villi are the major placental component. The later-stage villi were still positive for HtrA3, but their staining intensity was much weaker than in the first trimester (Fig. 1C). The most dramatic down-regulation was in the syncytiotrophoblast, in which HtrA3 was greatly reduced in the second and third trimesters (P = 0.0003, Fig. 1, C and D). Furthermore, villous syncytiotrophoblasts and cytotrophoblasts showed equal low HtrA3 expression in the second trimester. Cytotrophoblast HtrA3 was further decreased to nondetectable levels by the third trimester (Fig. 1C). The fetal blood vessels within third-trimester villi were positive for HtrA3 and of similar intensity to the corresponding syncytiotrophoblast (Fig.
1C). Levels in the decidua remained high throughout pregnancy (Fig. 1B).

The dynamic pattern of placental HtrA3 during pregnancy is reflected in maternal serum

HtrA3 is detectable in the maternal circulation during the first trimester (13). Because placental immunoreactive HtrA3 decreased with increasing gestation, we determined the serum HtrA3 as gestation increased. HtrA3 was clearly detectable in all sera examined, but levels were consistently higher in all first-trimester vs. third-trimester sera (Fig. 2A). A predominant band at approximately 39 kDa was detected in the first trimester, whereas two to three bands of similar intensity clustered at approximately 39 kDa in the third trimester (Fig. 2A). Densitometric analysis based on equal protein loading (Supplemental Fig. 1, published on The Endocrine Society’s Journals Online web site at http://jcem.endojournals.org) revealed that mean total HtrA3 in third-trimester sera was approximately 30% of that in the first trimester ($P = 0.0001$, Fig. 2B). Thus, the dynamic pattern of serum HtrA3 levels during pregnancy mimics that of placental HtrA3.

Maternal HtrA3 serum levels at the end of the first trimester differ between women who subsequently did or did not develop preeclampsia

Serum levels of HtrA3 in the first and/or third trimester were then measured in women who did or did not develop preeclampsia. HtrA3 ($\sim 39$ kDa) was clearly detected in all 10- to 14-wk samples. HtrA3 levels at similar gestation in women who subsequently did or did not develop preeclampsia were, although overlapping, significantly different at 13–14 wk, being consistently higher in the preeclampsia group ($P = 0.0047$, Fig. 3). Furthermore, at 14 wk, serum HtrA3 levels appeared to decrease in normals but not in the preeclampsia sera (Fig. 3A), maximizing the difference (Fig. 3A). Before 13 wk, abundant HtrA3 was detected in all sera but with no difference between groups (Supplemental Fig. 2). In third-trimester serum, HtrA3 levels were low and not different between women who had preeclampsia at the time and gestation-matched controls (data not shown).

Thus, although a similarly high level of HtrA3 is produced in the first trimester with production down-regulated by the third trimester in both normal and preeclamptic pregnancies, the timing of HtrA3 down-regulation is
different. This occurs normally late in the first trimester but appears to be delayed in women who subsequently develop preeclampsia.

**HtrA3 secretion from first-trimester placental villi is enhanced by lower oxygen tension**

To understand the potential mechanisms controlling the production and down-regulation of placental HtrA3, we examined the DNA sequence of human \( HtrA3 \) for consensus motifs and identified binding sites for redox-sensitive transcription factors (including nuclear factor-κB, activator protein 2, and hypoxia inducible factor 1) in the promoter region. We thus investigated whether oxygen tension regulates placental HtrA3 production/secretion.

When first-trimester placental villi were cultured as explants, a 39-kDa band of HtrA3 similar to that detected in first-trimester serum was evident in Western blots of culture media within 24 h (Fig. 4A). A significantly higher level of HtrA3 was detected in the media when the villi were cultured under 3% compared with 20% O\(_2\) (\( P = 0.039, \) Fig. 4), confirming that first-trimester villous HtrA3 secretion or production/secretion was sensitive to oxygen tension and enhanced by low oxygen.

**Syncytialization enhances HtrA3 protein secretion without affecting its expression**

Syncytiotrophoblast is the primary source of HtrA3 production in first-trimester villi (Fig. 1) and is largely responsible for placental secretion into maternal circulation. To establish that the observed effect of oxygen tension on HtrA3 production/secretion was a syncytiotrophoblast response, we examined the regulation of HtrA3 by oxygen tension in syncytiotrophoblast using a well-established BeWo cell syncytialization model in which syncytialization was induced with forskolin. Cellular E-cadherin was reduced greater than...
70% (Fig. 5), whereas β-hCG in the media increased approximately 7-fold (from 5500 to 39400 IU/liter) in forskolin-treated compared with untreated cells, confirming successful syncytialization.

When HtrA3 protein levels were compared between syncytial and nonsyncytial cells, a higher level in the media but a lower level in the cell lysates was detected in the syncytial cells (Fig. 5A). These reciprocal changes were of similar magnitude (Fig. 5B), suggesting that syncytialization enhanced HtrA3 secretion without affecting its total production. This finding was supported by no change in the HtrA3 mRNA (short form) level with syncytialization (Fig. 5C).

**HtrA3 protein is regulated by oxygen tension specifically in syncytiotrophoblast**

A cell model was used to prove the principle that oxygen tension may regulate HtrA3. Syncytial vs. nonsyncytial BeWo cells were cultured under 2.5 or 20% O₂ for 24 h and HtrA3 protein and mRNA levels compared. For nonsyncytial cells, oxygen tension affected neither cellular production nor secretion of HtrA3 protein nor HtrA3 mRNA expression (Fig. 6, A–C). In contrast, syncytial cells (Fig. 6, D–F) responded dramatically to oxygen tension. Cellular HtrA3 protein levels were significantly elevated under 2.5% compared with 20% O₂ (P = 0.025, Fig. 6E). HtrA3 mRNA levels also showed a trend toward increase under 2.5% O₂, although this was not significant (Fig. 6F).

To test whether changing oxygen tension from a high to a low concentration will affect HtrA3, syncytial and nonsyncytial cells were cultured under 2.5% O₂ for 24 h and then transferred to 20% O₂ for 8 h, and the HtrA3 protein and mRNA were compared. For nonsyncytial cells (Fig. 6, A–C), the increase in oxygen concentration had no significant effect on the HtrA3 protein production, secretion, or mRNA expression. In contrast, in syncytial cells (Fig. 6, D–F), a low to high oxygen switch significantly reduced both the cellular (P = 0.029) and secreted (P = 0.023) HtrA3 proteins. A similar trend, but not statistically significant change, was seen for HtrA3 mRNA, suggesting that the regulation is mainly at the level of protein production and/or stability. These results demonstrated a clear differential effect of oxygen tension on HtrA3 protein in the syncytial vs. nonsyncytial cells; lower oxygen tension increased, whereas a low to high oxygen switch substantially decreased the HtrA3 protein specifically in the syncytial cells.

**Discussion**

We have established that the serine protease HtrA3 is maximally produced in the placenta in the first trimester and then dramatically down-regulated, especially in syncytiotrophoblast. This is reflected in maternal serum levels. HtrA3 is regulated by oxygen tension specifically in syncytiotrophoblast, being enhanced by low oxygen and dramatically reduced when oxygen rises. This provides a strong molecular explanation for why HtrA3 is reduced in
the placenta and in maternal blood at the end of the first trimester when the low-to-high oxygen switch occurs \textit{in vivo}. Furthermore, we have demonstrated a clear correlation between maternal serum levels of HtrA3 at around the time of the oxygen switch and the risk of preeclampsia. Preeclampsia remains one of the most complex challenges in modern perinatal care. Although preeclampsia is accepted to be of a heterogeneous etiology, abnormal early placentation and defective remodeling of the maternal vessels are recognized as important factors in the initiation of the disease (6, 7, 9). Thus, the early detection of preeclampsia should be feasible if abnormalities in vessel remodeling could be identified early. Indeed, uterine artery Doppler ultrasound detects decreased uteroplacental perfusion before the clinical onset of preeclampsia (22), whereas the Doppler screening of uterine artery blood flow shows 60% sensitivity to predict preeclampsia (5).

The challenge has been to identify a biomarker(s) that will detect early placentation abnormalities associated with vessel remodeling.

HtrA3 is secreted into the maternal circulation and its serum profile reflects placentation production. Production and secretion of syncytiotrophoblast HtrA3, the likely major source of serum HtrA3, are sensitive to oxygen tension. Changes in oxygen tension caused by altered uteroplacental blood flow will be directly sensed by syncytiotrophoblast, which is bathed in blood and will be reflected in serum HtrA3 levels. The dynamic pattern of placentation HtrA3 production and secretion across gestation is in strong agreement with this concept. Accordingly, monitoring serum HtrA3 late in the first trimester, when major changes in oxygen tension normally occur, may identify abnormalities in vessel remodeling. Our experimental data strongly support this notion and indicates that approximately 13–14 wk of gestation is optimal for detection. It appears that it is not HtrA3 production but rather its down-regulation at the end of first trimester that is delayed in women destined to develop preeclampsia. We therefore propose that serum HtrA3 is closely related to oxygen tension in the placenta and that abnormal levels at the end of the first trimester may indicate the risk of preeclampsia.

A number of biomarkers have been studied for their potential utility in aiding early diagnosis of preeclampsia. Placental growth factor (PIGF) is highly expressed in the trophoblast, and although plasma PIGF in the second half of pregnancy is significantly attenuated in preeclamptic pregnancies (23, 24), the plasma PIGF assay had insufficient sensitivity and specificity for predicting preeclampsia (24). However, preeclampsia does not develop in all women with high sFlt-1 and low PIGF levels and does develop in some women with low sFlt-1 and high PIGF levels (25). Maternal circulating levels of soluble endoglin (sEng) or CD105, a truncated form of endoglin, a cell surface coreceptor for TGF-β1 and TGF-β3, are

![FIG. 6. HtrA3 protein is regulated by oxygen tension specifically in syncytiotrophoblast. Nonsyncytial (A–C) and syncytial (D–F) BeWo cells are shown.](https://academic.oup.com/jcem/article-abstract/96/2/403/2769504)

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significantly elevated 5–8 wk before the onset of preeclampsia (28), and sEng has been suggested as a potential second- or third-marker for preeclampsia (29).

The ratio of sFlt-1 and sEng to PIGF is a better prediction marker than any single measurement (28). Placental protein 13 is also reported as a useful first-trimester biomarker, especially when combined with Doppler ultrasound (30–32). Other factors that were evaluated for predicting preeclampsia included inhibin A, activin A, pregnancy-associated plasma protein A, hCG, leptin, IGF-I, and IGF-binding protein-1 (33). Angiotensin receptor agonistic autoantibodies and catechol-O-methyltransferase are also associated with preeclampsia and have diagnostic implications (34, 35). However, further studies are required to prove that these markers will provide a sufficiently accurate screening test for broad clinical use in predicting preeclampsia, especially well before the clinical onset (5, 33). The measurement of HtrA3 appears to indicate the impairment of vessel remodeling as early as 13–14 wk of gestation (well before the clinical onset of preeclampsia). Further studies are warranted to examine whether HtrA3 represents a new potential biomarker.

Abnormal HtrA3 production may also directly influence the pathogenesis of preeclampsia. A recent major advance in understanding preeclampsia physiology is the altered pro-angiogenic balance involving decreased PIGF and increased sFlt-1 and sEng (24, 25, 28, 36). During normal pregnancy, physiological levels of VEGF and TGF-β signaling in the vasculature maintain vascular homeostasis (7). In preeclampsia, the placenta secretes excess antiangiogenic sFlt1 and sEng, which inhibit VEGF and TGF-β signaling, respectively, causing imbalance and endothelial cell dysfunction (24, 25, 28, 36). Endoglin is a cell surface coreceptor for TGF-β1 and TGF-β3; sEng competes for TGF-β binding to its receptors, thereby inhibiting TGF-β signaling (28). Intriguingly, HtrA3, and its family member HtrA1, are inhibitors of TGF-β signaling (37–39). We thus speculate that abnormally high levels of HtrA3 may contribute to the pathogenesis of preeclampsia via inhibition of TGF-β signaling.

In summary, HtrA3 protein production is closely associated with oxygen tension in the placenta, and HtrA3 reduction at the end of the first trimester reflects the placental oxygen switch associated with vessel remodeling. We have provided evidence that abnormally high serum HtrA3 levels at approximately 13–14 wk of gestation may be associated with the risk of developing preeclampsia. Sensitive and high-throughput HtrA3 assays and large-longitudinal studies are required to determine the clinical utility of monitoring serum HtrA3.

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