Increased Apoptosis in First Trimester Extravillous Trophoblasts from Pregnancies at Higher Risk of Developing Preeclampsia

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Preeclampsia complicates 5 to 10% of pregnancies and is a leading cause of maternal and fetal mortality and morbidity. Although the cause is unknown, inadequate invasion and remodeling of maternal uterine arteries by extravillous trophoblasts (EVTs) in the first trimester is a common feature. Uterine spiral artery resistance as detected by Doppler ultrasound is commonly used in the second trimester to identify pregnancies destined to develop preeclampsia. Correlation between high uterine resistance and the failure of trophoblast invasion has been reported as early as 12 weeks. However, the reason for this failure has not been established. Understanding the processes involved would significantly improve our diagnostic potential. In this study, we correlated increased first trimester uterine artery resistance with a biological abnormality in trophoblast function. EVT's derived from high-resistance pregnancies were more sensitive to apoptotic stimuli than those from normal-resistance pregnancies. Survival of EVT's from high-resistance pregnancies could be increased by nitric oxide, whereas inhibition of nitric oxide in cells from normal-resistance pregnancies increased apoptotic sensitivity. This predates the onset of symptoms by several weeks and provides evidence for a mechanism responsible for the incomplete uterine vessel remodeling and the differences in artery resistance between preeclamptic and normal pregnancies. (*Am J Pathol* 2007, 170:1903–1909; DOI: 10.2353/ajpath.2007.070006)

Extravillous trophoblast cells (EVTs) detach from the anchoring placental villi to invade the uterine wall and its blood vessels as far as the inner myometrial segments.

Trophoblast invasion occurs during the first 20 weeks of pregnancy; during this period trophoblast cells, endothelial cells, and vascular smooth muscle cells transiently coexist in partially modified spiral arteries.¹ Access to maternal arteries by EVT's may be by endoluminal upstream migration or by *trans*-stromal migration and subsequent penetration of the arterial wall.^{2–6} As pregnancy progresses, EVT's surround and invade the artery walls, fibrinoid matrix accumulates, and musculoelastic tissue is lost.⁷ This creates a high-flow, low-resistance zone within the placenta where maximal exchange of nutrients, respiratory gases, and waste products between the maternal and fetal circulations can occur. Although it has been suggested that some changes in the vessels are independent of EVT's, occurring as part of the maternal response to pregnancy,⁸ there is also evidence of an active role for EVT's in artery remodeling,^{9–11} and in the absence of trophoblastic invasion, the extent of this vessel remodeling is radically curtailed.

Inadequate invasion and remodeling of the uterine spiral arteries has been associated with complications of pregnancy such as preeclampsia (PE), intrauterine growth restriction (IUGR), and spontaneous preterm birth. Although the mechanisms responsible for this have yet to be fully established, it is clear that factors affecting the balance between trophoblast proliferation and death may play an important role. Apoptosis occurs during tissue morphogenesis and homeostasis as an essential balance to cell replication and has been identified as part of normal placental development.^{12,13} Increased apoptosis has been observed in EVT's of placentas from pregnancies complicated by PE and IUGR.^{14,15} In subse-

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quent studies of preeclamptic pregnancies, this was found to be restricted to the endovascular rather than interstitial subpopulation EVTs.^{16,17}

Noninvasive assessment of uterine artery blood flow resistance using Doppler ultrasound indicates high-resistance blood flow in early pregnancy. After the transformation of the uterine spiral arteries, a low-resistance blood flow pattern develops. Many studies have demonstrated a correlation between uterine artery resistance and pregnancy outcome. Pregnancies with high resistance are more likely to develop PE and IUGR.^{18,19} More recent findings indicate that resistance indices obtained in the first trimester (weeks 11 to 14) correlate with birth weight and the extent of trophoblast invasion.^{20,21}

The aim of this study was to determine whether first trimester EVTs derived from pregnancies with high uterine artery resistance were more susceptible to apoptotic stimuli than those with normal uterine artery resistance and to investigate the mechanisms responsible for this.

Materials and Methods

Determination of Uterine Artery Resistance

Doppler ultrasound examination of the maternal uterine arteries was performed in women attending a clinic for termination of pregnancy in the late first trimester. Only singleton pregnancies were included. Women with a known medical condition (eg, diabetes mellitus, connective tissue disease, and essential hypertension) or a history of recurrent miscarriage were excluded from the study. Gestational age was calculated from the last menstrual period and confirmed by crown-rump length measurement. In all cases, a careful search for fetal abnormalities was performed, and 5% of samples were excluded. Local ethical committee approval was obtained for this study, and all women gave written, informed consent.

Examinations were performed using an Acuson XP-10 system (Mountain View, CA) equipped with a 5-MHz curvilinear transabdominal probe, following a previously described technique.²² The high-pass filter was set to the minimum, and the pulse repetitive frequency was 2.5 kHz. The maximum achievable thermal and mechanical indices were 1.2 and 1.0, respectively. The size of the sampling gate was set to 2 mm. A mid-sagittal section of the uterus was obtained, and the cervical canal was identified. The probe was then moved laterally until the paracervical vascular plexus was seen. Color Doppler was turned on, and the uterine artery was identified as it turned cranially to make its ascent to the uterine body. Measurements were taken at this point, before the uterine artery branched into the arcuate arteries. Once it was ensured that the angle was less than 60°, the pulsed Doppler gate was placed over the vessel. Angle correction was then applied, and the signal was updated until at least four consecutive flow velocity waveforms of good quality were obtained. The resistance index (RI) was calculated (systolic velocity – diastolic velocity/systolic velocity) and recorded, as was the presence or absence

of an early diastolic notch.²³ Measurements were obtained on the left and right side, and the mean RI was calculated.

Because there is no agreement in the literature as to whether early diastolic notching is an indicator of resistance independent from the RI, high uterine artery resistance cases were defined as those presenting with bilateral uterine artery notches and a mean RI above the 95th percentile, representing approximately 5% of the population. Normal-resistance cases were defined as those presenting with no uterine artery notches and a mean resistance index below the 95th percentile, representing approximately 40% of an unselected population. These groups represent those most (30%) and least (1%) likely to have developed PE if the pregnancy had progressed.^{20,24}

Chorionic Villous Explant Culture

Preparation of first trimester placental explants was performed as previously described.^{25–27} Those preparing the tissue were blinded to the clinical indices. Placental villous tissue was separated from the decidua and rinsed extensively with Hanks' balanced salt solution. Under a dissecting microscope, small pieces of tissue (2 to 3 mm) were removed from the periphery of the villous tree; to optimize the presence of anchoring villi, 50 to 100 villi were randomly selected and explanted from each placenta and placed on collagen rafts. Ham's F-12/Dulbecco's modified Eagle's medium (1:1) plus penicillin (100 U/ml) and streptomycin (0.1 mg/ml) was then added around the collagen raft, and the explants were incubated in 5% CO₂ in air at 37°C overnight to allow adhesion. After 16 hours of incubation, a further 2 ml of Ham's F-12/Dulbecco's modified Eagle's medium was added to each well, and nonadherent tissue was gently removed. During the following 6 days, cells migrated from the anchoring villi forming a monolayer. Immunostaining of parallel cultures for the trophoblast-specific marker cytokeratin-7 identified these cells to be at least 98% positive using the previously described method (Figure 1A).²⁸

Induction of Apoptosis

After migration of EVTs from villous explants (after 7 days of culture), tumor necrosis factor- α (TNF- α) and actinomycin D were added to the villous explants, which were then mounted on an Olympus IX70 inverted microscope (Olympus, Tokyo, Japan) equipped with a Hamamatsu C4742-95 digital camera and motorized stage (Hamamatsu Photonics, Hertsfordshire, UK). The microscope and stage were enclosed within a heated chamber (37°C), and cells were cultured in 5% CO₂ in air to maintain pH (Solent Scientific, Segensworth, UK). Images were captured every 15 minutes and analyzed using Image Pro Plus software (Media Cybernetics, Silver Spring, MD). At the beginning of each time-lapse sequence, at least 40 cells were identified and tracked. These cells were chosen at random and without regard for the distance they had migrated from the cell column.

Figure 1. Monitoring primary EVT apoptosis. **A:** The trophoblastic nature of the primary cells that migrate from chorionic villous explant cultures was confirmed by positive cytokeratin-7 staining of 7-day-old cultures. Parallel cultures were incubated in the absence (**B**) or presence (**C**) of TNF- α (30 ng/ml) and actinomycin D (200 ng/ml) and monitored by time-lapse microscopy for 12 hours. Typical morphological changes observed during the induction of apoptosis in these cells are depicted in **D**. The cell shown after 4 hours has become phase bright with characteristic blebs. After 5.5 hours, this cell has developed blisters. Scale bar = 50 μ m. Two short video sequences are available online (supplementary video at <http://ajp.amjpathol.org>).

The cells were scored according to the time at which clear apoptotic morphology was first observed. Apoptotic morphology was considered as cytoplasmic and nuclear shrinkage and a change to a phase bright appearance,

with the formation of membrane blebs/blisters. In previous studies in which cell numbers were not limiting, we have correlated these morphological changes with changes in biochemical markers of apoptosis such as caspase activation and cleavage of poly(ADP-ribose) polymerase.^{29–31} In this study, we confirmed that the stimuli used induced apoptosis by prior incubation of cells with the broad-spectrum caspase inhibitor zVAD-fmk (10 μ mol/L). In this series of experiments, apoptosis was induced by TNF- α (30 ng/ml) and actinomycin D (200 ng/ml) in Ham's F-10 containing 10% (v/v) fetal calf serum. A smaller series of patients was randomly chosen from each group ($n = 10$ normal resistance and $n = 9$ high resistance). The patients from these two subgroups were not significantly different for parity, gestational age, and uterine artery resistance indices from those used in the larger kinetic study. In this subgroup, EVT cultures were incubated with either 100 μ mol/L PAPA-NONOate added at the time of stimulation or 5 mmol/L *N*^w-nitro-L-arginine methyl ester added 24 hours before the apoptotic stimuli.

Statistical Analysis

Nonparametric statistical tests were used throughout this study. Patient demographics are expressed as the medians and ranges and analyzed for significance using the Mann-Whitney *U* test. The kinetics data are means $\pm$ SD of the mean and analyzed for significance using the Mann-Whitney *U* test. Correlation between uterine artery resistance and survival was determined using the Spearman Rank Correlation. A *P* value (all two-sided) of <0.05 was considered to be statistically significant.

Results

Thirty-three pregnancies were examined, 13 with high-resistance uterine artery Doppler indices and 20 with normal-resistance Doppler indices. The median maternal age was 27 years (range, 18 to 42); gravidity, 2 (range, 1 to 6); parity, 0 (range, 0 to 3); gestational age at ultrasound scan, 11.4 weeks (range, 10.0 to 13.4); and gestational age at sampling, 12.1 weeks (range, 10.1 to 13.6). The median interval between the ultrasound scan and sampling was 4 days (range, 1 to 10). No significant differences in clinical characteristics were found between the two groups (Table 1). Chorionic villous explants were

Table 1. Demographic and Clinical Characteristics in Pregnancies with High and Normal Resistance Uterine Artery Blood Flow

Patient characteristics	High resistance	Normal resistance	<i>P</i> value
<i>n</i>	13	20	
Maternal age (years)	28 (18 to 42)	26.5 (19 to 41)	0.24
Gravidity	3 (1 to 5)	2 (1 to 6)	0.30
Parity	0 (0 to 3)	0 (0 to 2)	0.55
Gestational age at scan (weeks)	11.1 (10.0 to 12.3)	11.85 (10.0 to 13.4)	0.16
Gestational age at sampling (weeks)	11.7 (10.1 to 12.9)	12.3 (10.6 to 13.6)	0.16
Scan to sampling interval (days)	4 (1 to 10)	4 (1 to 10)	0.95

The results are expressed as the median, with the range in brackets. Statistical significance was assessed using the Mann-Whitney *U* test, and significance was taken as $P < 0.05$.

Figure 2. Kinetics of apoptosis of EVT cells from pregnancies with high and normal uterine artery resistance. EVT cells isolated from patients who had undergone termination of pregnancy with normal uterine artery resistance (<95 th percentile, ■, $n = 20$) or high resistance (>95 th percentile, □, $n = 13$) were incubated with TNF- α (30 ng/ml) and actinomycin D (200 ng/ml). At least 40 cells were chosen at random, and their fate was followed by time-lapse microscopy. The time at which morphological changes characteristic of apoptosis developed was noted for each cell, and the mean $\pm$ SD of data from patients grouped according to resistance is shown in **A**. The $t_{1/2}$ was calculated for each patient sample, and the data from high uterine-resistance and the normal-resistance patients are combined in **B**. The mean ($t_{1/2}$) $\pm$ SD is shown with *** $P < 0.001$.

isolated from the products of conception, and the anchoring villous tips were mounted on collagen rafts. EVT cells migrated from the tips of the chorionic villous explants and by the 7th day formed a viable cell monolayer where individual cells are clearly visible by phase contrast microscopy (Figure 1). In parallel cultures, these cells were shown to stain positive for cytokeratin-7 (Figure 1) and were mononuclear as determined by 4,6-diamidino-2-phenylindole staining (data not shown). In the absence of actinomycin D, TNF- α had no significant effect on the induction of trophoblast apoptosis (data not shown). After 12 hours of culture with TNF- α /actinomycin D, all of the EVT cells had undergone apoptosis (Figure 1C), as determined by exhibition of characteristic apoptotic morphology, including cytoplasmic shrinkage, a phase bright appearance, and membrane blebbing and blistering (Figure 1D; supplementary video at <http://ajp.amjpathol.org>). A significant advantage of using time-lapse microscopy in this study was that the kinetics of induction of apoptosis in EVT cells could be determined using these cells (Figure 2A). As all of the cells identified for analysis at the start of the experiment underwent apoptosis over the 12-hour period, we were able to determine the time at which 50% of the cells had died and termed this the $t_{1/2}$ value. Incubation of explant cultures with zVAD-fmk before the addition of the TNF- α /actinomycin D reduced the number of cells expressing the characteristic apoptotic morphology from 96.7 ± 4.7 to $2.5 \pm 3.1\%$ ($n = 6$), indicating that cell death was caspase-dependent. This result was independent of patient group. In addition, detection of fragmented nuclei using 4,6-diamidino-2-phenylindole staining and positive immunostaining for cleaved poly(ADP-ribose) polymerase in treated but not untreated cells derived from explant cultures confirmed apoptosis (data not shown).

The mean $t_{1/2}$ for the high-resistance pregnancies was 3.91 ± 0.24 hours ($n = 13$), whereas that obtained from pregnancies with normal uterine artery resistance was 5.8 ± 0.8 hours ($n = 20$). This difference in the time taken to induce apoptosis between the two populations was

Figure 3. Correlation between apoptosis and uterine artery resistance. The uterine artery resistance index was plotted against the time at which 50% of the cells had undergone apoptosis ($t_{1/2}$, obtained from the individual kinetics curves). A significant inverse correlation of -0.82 (Spearman correlation), $P < 0.001$ was obtained. These data were derived from 33 patients, and some data points were coincident.

highly significant, $P < 0.001$ (Figure 2B). To analyze these data further, we examined the possible correlation between a measure of uterine artery resistance, the RI, and the $t_{1/2}$ values (Figure 3). Using the Spearman correlation, a significant inverse correlation was obtained between the RI and the $t_{1/2}$ ($r = -0.82$, $P < 0.001$), indicating that a higher resistance index correlated with a reduction in EVT survival. Gestational age had no significant effect on the sensitivity to apoptosis found within each group.

We have previously demonstrated that nitric oxide (NO) plays an important role in regulating the sensitivity of EVT cells to apoptotic signals.^{29,30} We therefore used the NO donor PAPA-NONOate and the NO synthase inhibitor *N*^ω-nitro-L-arginine methyl ester to investigate the role played by NO in determining the differences in sensitivity to apoptotic signals exhibited by these two populations. Cultures derived from high uterine artery-resistance pregnancies were significantly less sensitive to the induction of apoptosis after the addition of the NO donor PAPA-NONOate ($P < 0.001$, $n = 5$), whereas inhibition of NO in cells from the normal population made them significantly more sensitive to apoptotic stimulation ($P < 0.05$, $n = 5$; Figure 4). Inhibition of NO synthesis had no significant effect on the sensitivity of high-resistance populations ($P > 0.05$, $n = 5$), although the addition of the NO donor to the normal-resistance population did have a small but significant further increase in the $t_{1/2}$ ($P < 0.05$, $n = 5$).

Figure 4. Effect of NO on EVT apoptosis. EVTs isolated from high- and low-resistance pregnancies ($n = 5$ patients each group) were incubated with TNF- α (30 ng/ml) and actinomycin D (200 ng/ml) alone, $\pm$ the NO donor PAPA-NONOate (100 μ M) or $\pm$ the NO synthase inhibitor N^G -nitro-L-arginine methyl ester (5 mmol/L). The time at which 50% of the cells became apoptotic was determined and compared with TNF- α /actinomycin D alone. Open bars represent EVTs from pregnancies with high resistance, and the filled bars are EVTs from normal-resistance pregnancies. The data are expressed as the mean $\pm$ SD of $n = 5$ patients for each group.

Discussion

Preeclampsia is a leading cause of maternal deaths in the developed world, which threatens the lives of thousands of women and their babies and places a significant burden on health care systems worldwide. Despite the severity and frequency of this disorder, the pathophysiology of the condition is poorly understood. An early event in establishing a healthy pregnancy is the invasion and remodeling of the maternal spiral arteries by EVTs. High uterine artery resistance as determined by Doppler ultrasound in the second trimester is associated with a reduction in trophoblast invasion.³² High uterine artery resistance as early as 10 to 14 weeks of gestation correlates with both low birth weight and a histological measure of trophoblast invasion.^{20–22} A number of studies report that high uterine artery resistance correlates with poor pregnancy outcome, including PE, IUGR, and spontaneous preterm delivery.^{18,19,24,33} However, the relative risk for developing PE is severalfold greater than for the other pathologies, including IUGR.^{18,19,24,33} Although those pregnancies with high first trimester uterine artery resistance exhibit reduced trophoblastic invasion, the

biochemical and cellular basis for the failure of EVTs to invade and remodel the uterine spiral arteries has yet to be established. The data from this study demonstrate that invading EVTs from pregnancies at increased risk of PE are more likely to undergo apoptosis during culture than from normal pregnancies. To improve the discriminatory power of the experiments, pregnancies with the lowest and highest risk of PE were compared. However, sensitivity to apoptosis shows a linear relationship with a quantitative index of uterine artery resistance such as RI. Supplementation of NO desensitizes the cells in the group with high uterine artery resistance to apoptotic stimuli, whereas blockade of NO increased death in the normal group.

Changes within the developing placenta require stringent spatial and temporal regulation of processes such as proliferation and apoptosis. Apoptosis has been identified in the placenta associated with endothelial, trophoblast, and stromal cells.^{12,13} Pro- and anti-apoptotic proteins are differentially expressed in syncytial and cytotrophoblasts throughout gestation, indicating that their susceptibility to apoptotic signals at different times of gestation may vary and could be important for normal placental development. The mechanisms involved in the regulation of trophoblast survival are poorly understood. Although increased apoptosis has been observed in EVTs from term pregnancies complicated by PE and IUGR,^{14–17} the high frequency of these apoptotic events in the study by DiFederico et al¹⁵ has been questioned.¹⁶ In this later study, M30 staining, which is specific for caspase-dependent apoptosis in epithelial cells, rather than TUNEL staining was used. Using this protocol staining for apoptotic cells was restricted to endovascular EVTs, whereas contrary to expectations, the rate of apoptosis in the interstitial trophoblasts was reduced in placenta of PE pregnancies compared with normal controls.^{16,17} Whether these results can be extrapolated to the first trimester when the events leading to PE are initiated is not known. Certainly, increased apoptosis in the endovascular EVTs population in the first trimester could result in reduced spiral artery remodeling.

TNF- α is an inflammatory cytokine and, together with other apoptotic cytokines, it is found at the utero-placental interface in early pregnancies.³⁴ Placental expression and the circulating concentration of TNF- α are elevated in pregnancies complicated by preeclampsia compared with normal pregnancies.^{35–38} In one study, TNF- α was found to be elevated early in pregnancy in women who later developed preeclampsia³⁹; moreover, TNF- α has been implicated in the inhibition of endovascular trophoblast invasion.^{40,41}

TNF signaling is complex and regulated by the expression of a number of pro- and anti-apoptotic factors. Despite expressing both TNF- α and its receptor, EVTs remain resistant to the induction of apoptosis by these molecules,⁴² suggesting, that under normal circumstances, the balance is in favor of survival. Actinomycin D is commonly used as an inhibitor of gene expression to shift the balance in favor of apoptotic signaling. NO is an important signaling molecule that regulates a plethora of physiological processes, including vasodilatation, inflam-

mation, and cell death and survival. NO is involved in pregnancy through its action as a vasodilator and its role in regulating trophoblast functions such as implantation,⁴³ differentiation,⁴⁴ motility, and invasion.⁴⁵ EVT's express inducible NO synthase and endothelial NO synthase.^{46–48} We have previously reported that the onset of apoptosis induced by TNF- α and actinomycin D in first trimester EVT's was delayed by prior stimulation with hepatocyte growth factor. This effect could be reversed by inhibiting NO production and mimicked by the addition of exogenous NO.³¹ Because these studies demonstrated the importance of NO in regulating trophoblast survival, we tested the hypothesis that it is a lack of NO production in the EVT's from high-resistance pregnancies that is responsible for their increased sensitivity to apoptotic stimuli. As hypothesized, inhibiting NO synthesis had no further effect on the sensitivity of the high-resistance EVT's to apoptotic stimulation, probably reflecting the maximum rate at which death can be induced in these cells through a receptor-mediated mechanism. However, supplementation with NO reduced their sensitivity to stimulation. Conversely, inhibition of NO increased the rate of apoptosis in cells from pregnancies with normal uterine artery resistance. The addition of NO to these cells increased their resistance to apoptotic stimuli, suggesting that in these cells, their sensitivity to apoptosis could be modulated in a positive and a negative way by altering endogenous NO production. The inhibitor studies clearly demonstrate a functional role for NO produced by EVT's.

NO synthesis can be regulated in a number of ways, including substrate and cofactor availability, enzyme expression, and intracellular concentration of the endogenous competitive inhibitors of NO synthesis, asymmetric dimethylarginine (ADMA) and mono methylarginine. There is a fall in the circulating concentration of ADMA in the first trimester of normal pregnancy,⁴⁹ whereas we and others have demonstrated an increase in maternal serum ADMA when the pregnancies are complicated by PE.^{49–51} More recently, raised plasma ADMA in the second trimester has been associated with increased bilateral uterine artery resistance and the onset of PE.⁵² It is therefore interesting to speculate that the increased sensitivity to apoptotic stimuli observed in the high-resistance cells could be due to decreased NO synthase activity. However, because of the limited number of cells available and the insensitivity of existing assays, intracellular ADMA, NO production, or NO synthase activity could not be detected in this study. However, in this regard, it is of note that a recent study has suggested that arginine supplementation can relieve the maternal symptoms of PE⁵³ and perhaps that a safe and simple intervention can also be used early in pregnancy to reduce the sensitivity of trophoblasts to apoptotic stimuli and improve uterine remodeling.

In conclusion, even though it is known that high uterine artery resistance is associated with reduced remodeling of the maternal spiral arteries, the mechanisms responsible for this are unknown. In this study, we present evidence that first trimester EVT's from pregnancies with high uterine artery resistance are inherently more sensitive to apoptotic stimuli and therefore worthy of clinical

attention. Furthermore, we provide evidence that abnormality may lie in the ability of the trophoblasts to produce NO. It is clear, however, that increased trophoblast sensitivity to apoptotic stimuli alone is not sufficient for PE to develop, because 70% of pregnancies with high uterine artery resistance proceed normally to term.²⁴ We therefore propose a two-component model for the development of PE requiring, on the fetal side, increased trophoblast sensitivity to apoptotic signals due in part to reduced NO synthesis and, on the maternal side, increased production of apoptotic stimuli such as TNF- α produced by decidual immune cells such as macrophages.⁴⁰ In future studies, it would be intriguing to determine whether there is a correlation between high uterine artery resistance and increased maternal production of apoptotic cytokines such as TNF- α .

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