Preeclampsia Is Associated with Widespread Apoptosis of Placental Cytotrophoblasts within the Uterine Wall

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Preeclampsia is a serious pregnancy complication diagnosed by signs of widespread maternal endothelial dysfunction. In normal pregnancy, a subpopulation of placental cytotrophoblast stem cells executes an unusual differentiation program that leads to invasion of the uterus and its vasculature. This process attaches the conceptus to the uterine wall and starts the flow of maternal blood to the placenta. Preeclampsia is associated with abnormal cytotrophoblast differentiation, shallow invasion, and decreased blood flow to the placenta. To determine whether abnormal differentiation and/or hypoxia leads to cytotrophoblast apoptosis, we used the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) method to label DNA strand breaks in tissue sections of the placenta and the uterine wall to which it attaches. Control samples ($n = 9$) showed almost no apoptosis, but in samples from patients with preeclampsia, 15–50% of the cytotrophoblasts that invaded the uterine wall were labeled (8/9 samples). These same cells failed to stain for Bcl-2, a survival factor normally expressed by trophoblasts in both the placenta and the uterine wall. Our results show that preeclampsia is associated with widespread apoptosis of cytotrophoblasts that invade the uterus. The magnitude of programmed cell death in this population may account for the sudden onset of symptoms in some patients, as well as the associated coagulopathies. (*Am J Pathol* 1999, 155:293–301)

The preeclampsia syndrome affects approximately 7% of nulliparous women.¹ The mother shows signs and symptoms that suggest widespread alterations in endothelial function (eg, high blood pressure, proteinuria, and edema).² In some cases the fetus stops growing, which leads to intrauterine growth retardation. The dangers of this condition are exacerbated by the fact that the maternal and fetal signs can suddenly appear at any time from

mid-second trimester until term—hence the name preeclampsia (from Greek *eklampsis*, sudden flash or development).

Both the etiology and the only known cure for this condition involve the placenta. One of the most important risk factors is an increase in placental mass. As a result, women carrying multiple fetuses are prone to development of this syndrome.³ Preeclampsia also can occur in hydatidiform mole, a condition in which genetically abnormal placental tissue (eg, trophoblast) proliferates in the absence of a fetus.⁴ In all cases the only known cure is removal of the placental tissue. If this is done before term, however, it can cause iatrogenic prematurity, further contributing to the morbidity and mortality associated with preeclampsia.

The placenta's role in preeclampsia has been enigmatic. Microscopic analyses of placental specimens from affected patients show that the cellular composition of floating chorionic villi—the subpopulation that floats in maternal blood and mediates gas and nutrient exchange—is relatively unaffected. In contrast, anchoring chorionic villi—the subpopulation that anchors the placenta to the uterine wall—show distinct anomalies.^{5–8} Normally, the invasive cytotrophoblasts that emanate from these anchoring villi are found in abundance throughout the interstices of the endometrium and the first third of the myometrium. In addition, they deeply invade the uterine spiral arterioles and open the superficial portions of the associated veins, a process that initiates flow of maternal blood to the placenta. In preeclampsia, the interstitial component of invasion is variably compromised, with abnormally shallow invasion most often associated with the appearance of signs in early gestation (Zhou and Fisher, unpublished results). But endovascular invasion is consistently rudimentary. As a result, the flow of oxygenated blood to the fetal-placental unit is reduced.

Our laboratory has been studying the differentiation pathway that normally leads to cytotrophoblast invasion and the defects in this process that are associated with

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preeclampsia. Knowledge of the cells' ability to intricately switch their adhesion molecule expression during the invasion process has been instrumental to the progress we have made. Thus far we know that, as part of the differentiation pathway that normally leads to endovascular invasion, cytotrophoblasts down-regulate the expression of adhesion molecules that are indicative of their epithelial origin (eg, E-cadherin, integrin $\alpha 6 \beta 4$) and up-regulate the expression of those that are important for endothelial cell function (eg, VE-cadherin, integrin $\alpha V \beta 3$, $\alpha 1 \beta 1$).⁹ In preeclampsia most aspects of this transition fail to occur, and undifferentiated, epithelial-like cytotrophoblast stem cells are found within the uterus.¹⁰ Recently we discovered that culturing normal cytotrophoblasts in a hypoxic atmosphere has the unusual effect of causing them to enter the cell cycle; this occurs at the expense of some aspects of the differentiation process, including the ability to up-regulate integrin $\alpha 1 \beta 1$ expression and their own invasiveness.^{11,12} This finding suggests one possible mechanism by which a reduction in maternal blood flow to the placenta could contribute to the altered placental phenotype associated with preeclampsia. Here we investigated the consequences of the aberrations we observed by testing the hypothesis that in preeclampsia, the presence of abnormally differentiated fetal cytotrophoblasts among the resident maternal cells of the uterus triggers apoptosis of one or both populations.

Materials and Methods

Placental Tissue Sources

Placental bed biopsy specimens were collected by direct visualization of the placental attachment site. Chorionic villi with attached decidua were dissected from three to five randomly chosen placental sites immediately after elective terminations or delivery. Nine control samples were obtained from women who were between 26 and 40 weeks of gestation. Of these, two samples were obtained from women who were delivered at 26 weeks, one because of cervical incompetence and the other because of inoperable conjoined twins. Seven specimens were obtained from control nulliparous women who underwent Cesarean sections at 33 (one), 34 (one), 35 (one), or 38 weeks of gestation (one) or delivered spontaneously at 39 (two) or 40 weeks (one). None of the control subjects had evidence of preeclampsia, gestational hypertension, chorioamnionitis, or chronic hypertension or a medical history that suggested they were at risk for developing preeclampsia.

Nine samples were obtained from preeclamptic patients at 26–39 weeks of gestation. Preeclampsia was diagnosed according to the following criteria, recommended by Chesley¹³: nulliparity; no history of hypertension before pregnancy; increase in diastolic pressure of 15 mm Hg or systolic pressure of 30 mm Hg compared with blood pressure obtained before 20 weeks of gestation; proteinuria ≥ 0.3 g/24 hours (or 1+ on urine dipstick) in a catheterized specimen; hyperuricemia >5.5 mg/dl

(or one SD greater than the normal mean value before term); and return to normal blood pressure and resolution of proteinuria by 12 weeks postpartum. Severe preeclampsia was diagnosed according to the following criteria, recommended by the American College of Obstetrics and Gynecology: systolic blood pressure ≥ 160 mm Hg and/or diastolic pressure ≥ 110 mm Hg; proteinuria of ≥ 5 g in a 24-hour period or 3+ on urine dipstick; and presence of cerebral or visual disturbances. Seven patients were diagnosed with severe preeclampsia and were delivered by Cesarean section (one each at 27, 28, and 31 weeks; two at 26 and 32 weeks); two with preeclampsia had vaginal deliveries (38 and 39 weeks).

Detection of Apoptotic Cells

Samples were processed immediately after they were obtained. The tissues were fixed in 3% paraformaldehyde for 30 minutes, infiltrated with 5–15% sucrose, embedded in optimal cutting temperature compound, and frozen in liquid nitrogen as previously described.¹⁴ Five to seven sections from three separate tissue blocks were used for detection of apoptosis and immunostaining.

Apoptotic cells were identified by the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) method, a commercial kit that fluorescein-labels DNA strand breaks (Boehringer-Mannheim, Indianapolis, IN). To identify trophoblasts among other fetal and maternal cells, TUNEL-stained frozen sections were double stained with a rat monoclonal antibody that specifically reacts with cytokeratin. The antibody was produced in this laboratory by using purified human cytotrophoblasts as the immunogen.¹⁵ Antibody binding was detected by using a rhodamine-conjugated secondary antibody as previously described.¹⁴ To identify immune and decidual cells, we used primary antibodies that specifically react with either CD45 (DAKO, Carpinteria, CA) or prolactin (Zymed, South San Francisco, CA), respectively, and the species-appropriate secondary antibodies.

The sections were then viewed with a Zeiss Axiophot epifluorescence microscope equipped with filters to selectively view the rhodamine and fluorescein images with no cross-contamination. The number of apoptotic nuclei, observed at a magnification of $\times 400$ with an oil immersion lens, was expressed as a percentage of the total number of cytokeratin-positive cells examined from each slide (300–1000). Statistical significance of the data was determined by using Student's paired *t*-test.

Cytotrophoblast nuclear morphology was also assessed by staining with Hoechst 33342 (Molecular Probes, Eugene, OR). After tissue sections were labeled with anti-cytokeratin, they were rinsed in buffer and placed in the dye (10 μ g/ml phosphate-buffered saline) for 2 minutes. After rinsing, immunoreactivity was assessed as described above. Hoechst staining was photographed under ultraviolet illumination.

Detection of Mitotic Cells

Sections immediately adjacent to those used for the detection of apoptotic cells were double stained (1 hour at

room temperature) with a mixture of antibody against a specific cell cycle marker and rat anti-cytokeratin. The former included mouse anti-Ki67 (1:200, v/v; Novocastra Laboratories, Newcastle on Tyne, UK), mouse anti-cyclin A (H432) (2 μ g/ml; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-cyclin B (D-11) (2 μ g/ml; Santa Cruz Biotechnology), and rabbit anti-phosphohistone H3 (2 μ g/ml; Upstate Biotechnology, Lake Placid, NY). Antibody binding was detected by using the appropriate secondary antibody, and samples were examined as described above.

Detection of Bcl-2-Expressing Cells

Sections immediately adjacent to those used for the detection of apoptotic cells were double stained (2 hours at room temperature) with a mixture of mouse anti-Bcl-2 (5 μ g/ml; Oncogene Research Products, Cambridge, MA) and rat anti-cytokeratin. Antibody binding was detected and samples were examined as described above.

Results

Invasive Cytotrophoblasts Undergo Apoptosis, but Not Mitosis, in Preeclampsia

In floating villi from either sample population, there was no evidence of apoptotic nuclei in trophoblast cells; a few stromal cells ($\leq 1\%$) in the villus cores were randomly labeled (data not shown). Likewise, in control samples the cytotrophoblast population that arose from anchoring villi and invaded the uterine wall showed very little apoptosis. Figure 1 is typical of the results we obtained. A 26-week sample from the control group contained an anchoring villus with abundant cytokeratin-positive cytotrophoblasts below the site of uterine attachment (Figure 1A). None of these fetal cells reacted with TUNEL (Figure 1B). Typically, a few cytokeratin-negative cells per field were labeled. This is in accord with the work of other investigators who have detected relatively few apoptotic cells in the human placenta from the first trimester onward.¹⁶

A tissue sample with anchoring villi that was obtained from a patient diagnosed with severe preeclampsia at 26 weeks of gestation also contained numerous cytokeratin-positive cytotrophoblasts (Figure 1C). As we and others have previously described,^{5,8,10} invasion was limited to the superficial portion of the uterus. TUNEL labeling showed evidence of widespread apoptosis among the cytotrophoblasts in this sample (Figure 1D) and in another specimen from a patient diagnosed with this syndrome at 31 weeks of gestation (Figure 1F). In these two samples and in one other specimen obtained at 28 weeks (data not shown), we also observed widespread apoptosis of cells that did not express cytokeratin. We subsequently investigated the identity of these cells by staining adjacent sections with antibodies that recognized either immune (anti-CD45) or decidual cells (anti-prolactin). The

cytokeratin-negative cells that labeled in Figure 1D expressed CD45 and therefore were primarily derived from the bone marrow, as were the cells in the 28-week specimen (data not shown). In contrast, the cytokeratin-negative cells that labeled in Figure 1F expressed neither CD45 nor prolactin. Thus we could not determine whether they were derived from yet another cell lineage or were apoptotic cells that had degraded the marker proteins we used for identification. In the remaining samples from preeclamptic patients, only apoptotic cytotrophoblasts in the uterine wall were observed.

Despite the intense nuclear staining of invasive cytotrophoblasts in severe preeclampsia, many of the TUNEL-labeled cytotrophoblast nuclei had relatively normal sizes and shapes, suggesting that they were in the initial stages of apoptosis (inset, Figure 1D). Somewhat fewer labeled cells had condensed, fragmented nuclei (Figure 1F). Hoechst staining demonstrated a similar spectrum of nuclear morphologies (Figure 2). Whereas the nuclei of some cytokeratin-positive cells had a normal appearance (Figure 2B), many others showed evidence of either chromatin condensation (Figure 2, D and F) or fragmentation (Figure 2, H and J). Previous reports suggest that apoptotic cells are recognized, ingested, and degraded beyond histological recognition in 1–2 hours.^{17,18} Thus, our data likely suggest that preeclampsia is associated with the sudden onset of widespread apoptosis of invasive cytotrophoblasts, and sometimes maternal cells, within the uterine wall.

Figure 3 summarizes the number of apoptotic (TUNEL-labeled) invasive cytotrophoblasts that were detected in tissue samples obtained from control ($n = 9$) and preeclamptic ($n = 9$) patients at 26–40 weeks of gestation. Little or no apoptosis was observed in the control samples until term, when most of the invasive cytotrophoblasts with labeled nuclei were localized in the deeper portions of the decidua (data not shown). In contrast, substantial apoptosis (mean $30.1\% \pm \text{SD } 17.9\%$ of cytotrophoblasts) was observed in eight of nine samples when the pregnancy was complicated by preeclampsia ($P \leq 0.001$). There was no correlation with gestational age; the two highest values, 53% and 54%, were obtained by analysis of samples obtained at 27 and 38 weeks of gestation, respectively.

It should be noted that one sample from a patient with preeclampsia did not show high levels of apoptosis, although the TUNEL-labeled cells were again observed at the invasion front. There could be several explanations for this, including the fact that the invasive trophoblast population found in this biopsy was in a different stage of apoptosis; not all phases are detected with TUNEL staining. Nevertheless, in this sample, as in the others from preeclampsia patients, invasive cytotrophoblasts failed to properly invade uterine arterioles; interstitial invasion was also abrogated compared to control specimens.

Because other investigators have reported enhanced cytotrophoblast proliferation in preeclampsia,¹⁹ we also examined this issue. We localized Ki67 (a nuclear antigen associated with proliferation/S phase), cyclin A (G1-S

Figure 1. Preeclampsia is associated with a large increase in TUNEL labeling of cytotrophoblasts (CTBs) within the uterine wall. Sections of placental bed biopsy specimens were from the following patients: (A, B) control subject at 26 weeks of gestation (26 W, CON); (C, D) patient with severe preeclampsia at 26 weeks of gestation (26 W, SPE); (E, F) patient with severe preeclampsia at 31 weeks of gestation (31 W, SPE). Sections were stained with anti-cytokeratin (CK: A, C, E) to identify CTB and were labeled by the TUNEL method (T: B, D, F) to detect cells undergoing apoptosis. In contrast to control samples, many of the cytotrophoblasts in the uterine walls of patients with severe preeclampsia were labeled with TUNEL. Often the nuclei appeared to be relatively intact (D, inset) although clusters of cells with fragmented nuclei were also seen (F, arrows). In 3/9 specimens, we also observed apoptosis in cells that did not express cytokeratin. Staining of adjacent sections with anti-CD45 showed that the smaller labeled nuclei in D (arrows) were those of immune cells (data not shown). In other cases, cells with fragmented nuclei (F, arrowheads), failed to stain with antibodies that specifically react with trophoblast, immune, or decidual cells.

CYTOKERATIN HOECHST 33342

Figure 2. Chromatin staining of cytotrophoblasts shows cytotrophoblasts in all stages of apoptosis. Sections from a 31-week placental bed biopsy sample of a patient with severe preeclampsia were stained with anti-cytokeratin (A, C, E, G, I) to identify cytotrophoblasts and Hoechst 33342 (B, D, F, H, J) to demonstrate chromatin. Whereas the nuclei of some cells had diffuse chromatin staining, others showed evidence of condensation (D, F) and fragmentation (H, J).

marker), cyclin B (G2-M marker), and phosphohistone H3 (mitosis marker) in all of our control and preeclampsia samples (data not shown). We saw no difference in the number of mitotic cytotrophoblasts in the two groups, which in all cases was very low. Nevertheless, the pos-

Figure 3. Percentage of TUNEL-labeled invasive cytotrophoblasts in tissue samples obtained from control ($n = 9$) and preeclamptic ($n = 9$) patients at 26–40 weeks of gestation. Each data point corresponds to the percentage of cytotrophoblasts (mean $\pm$ SEM) within the uterine wall that were labeled with TUNEL. Percentages were calculated by examining five to seven sections from at least three separate tissue blocks (see Materials and Methods).

sibility exists that enhanced proliferation precedes apoptosis and that different endpoints are detected in samples obtained at different stages during the disease process.

Invasive Cytotrophoblasts Fail to Express Bcl-2 in Preeclampsia

Next, we used sections cut from these same tissue samples to determine whether preeclampsia is associated with a change in expression of Bcl-2, an oncoprotein that can suppress programmed cell death in both normoxic and hypoxic conditions.^{20,21} In preliminary experiments we proved antibody specificity (data not shown); immunoblot analysis of placental villus lysates showed that the anti-Bcl-2 monoclonal antibody we used reacted with a single band of the expected molecular weight (M_r 24,000–26,000). We then used this antibody to stain sections of floating villi found in the placenta proper. As has been shown by other investigators,²² in control samples intense immunoreactivity was detected in association with both the cytotrophoblast layer that is attached to the trophoblast basement membrane and the overlying fused syncytiotrophoblasts (Figure 4B). This pattern did not change when the placental sample was obtained from a pregnancy complicated by preeclampsia (Figure 4D). We next examined Bcl-2 expression by invasive cytotrophoblasts that were found within the uterine wall, ie, the population in which a significant number of cells in preeclamptic samples were undergoing programmed cell death. In control pregnancies, groups of cytotrophoblasts stained intensely; a few cytotrophoblasts ($\leq 20\%$) did not react with the anti-Bcl-2 antibody (Figure 5B). In contrast, no staining above background was detected in cytotrophoblasts that invaded the uteri of patients with preeclampsia (eg, Figure 5D). Likewise, cytotrophoblasts in the sample from the preeclampsia

Figure 4. The anti-Bcl-2 staining pattern of trophoblasts in floating chorionic villi does not change in preeclampsia. Sections of placental bed biopsies were obtained from (A, B) a control subject at 26 weeks of gestation (26 W, CON) and (C, D) a patient with severe preeclampsia at 26 weeks of gestation (26 W, SPE). Sections were double stained by using anti-cytokeratin (CK; A, C) to identify both cytotrophoblasts (CTB) and syncytiotrophoblasts (ST) and anti-Bcl-2 (B, D) to identify cells that expressed this oncoprotein, which is also a survival factor. Cells that composed the villus core (VC) did not react with anti-Bcl-2, whereas the trophoblast layers stained brightly in all cases.

patient that showed low levels of apoptosis failed to express Bcl-2.

Discussion

Our finding that fetal cytotrophoblasts within the uterine walls of preeclamptic patients undergo programmed cell

death fits into the current paradigm of how apoptosis functions *in vivo*. Namely, one important purpose of this process is the selective deletion of abnormally differentiated cells that are, consequently, functionally impaired.²³ Our previous work has shown that preeclampsia is associated with abnormalities in the differentiation pathway that leads to cytotrophoblast invasion of the uterus and

Figure 5. The anti-Bcl-2 staining pattern of invasive cytotrophoblasts (CTB) is selectively reduced in preeclampsia. We studied sections of placental bed biopsies obtained from the same patients as the floating villi samples analyzed in Figure 3. (A) The CK-positive CTB within the uterine wall (B) normally stained brightly with anti-Bcl-2. (C) In contrast, CK-positive CTB in the uterine wall of a patient with severe preeclampsia (D) failed to react with this antibody, suggesting greatly reduced Bcl-2 expression.

the associated portions of maternal vessels. Specifically, the cells' repertoire of adhesion molecules is significantly altered from that observed in normal pregnancy,^{8,10} suggesting that their interactions with maternal cells and uterine extracellular matrix molecules are also abnormal. As has been observed in other systems,^{24,25} such anomalies can transmit intracellular signals that lead to apoptosis rather than survival.

Because many apoptotic cells appear to enter the cell cycle,^{26–28} the unusual effects of low oxygen on cytotrophoblast mitotic activity could also be relevant to our finding that these cells undergo apoptosis in preeclampsia. Before the cytotrophoblasts reach a supply of maternal blood, they proliferate in the hypoxic environment, near the uterine lumen, of the placenta proper. Within the uterine wall they stop dividing and differentiate along gradients of increasing oxygen tension, which we postulate helps to direct them toward maternal arterioles. Recently we modeled *in vitro* the situation that happens in preeclampsia. When the cells were confronted with an extracellular matrix in an hypoxic environment, they continued to proliferate while they differentiated, albeit abnormally.¹² We hypothesize that prolonging this situation, the likely scenario in preeclampsia, could eventually lead the cells to exit the cycle in G₁, directing them toward apoptosis rather than passage into S and mitosis. It would be of great interest to know when, during pregnancy, this cascade of events is initiated. In an attempt to answer this question we collected 100 chorionic villous samples, but none contained the population of invasive cytotrophoblasts that undergo apoptosis in preeclampsia.

Finally, our findings could help explain several well-recognized clinical aspects of this syndrome. For example, preeclampsia is associated with fibrin deposition at the maternal-fetal interface.²⁹ Recent data suggest that phosphatidyl serine, a neoantigen on the surface of apoptotic cells, has potent procoagulant activity.³⁰ Thus it seems likely that cytotrophoblasts undergoing programmed cell death could elicit fibrin deposition, as well as platelet activation, another common feature of preeclampsia.³¹ It remains to be determined whether this phenomenon is also relevant to the fact that women with anti-phospholipid antibodies have an increased risk of developing preeclampsia.³² Likewise, we do not know whether the effects we observed are limited to the chorion frondosum or spread to the chorion laeve. Currently, we are collecting the appropriate tissue samples to answer this question.

Another unique aspect of the clinical presentation of preeclampsia is its sudden appearance, particularly in patients with the most severe signs. Our findings suggest that the fetal cytotrophoblasts in direct contact with resident uterine cells are undergoing programmed cell death without a compensatory increase in mitosis. As a result, the maternal-fetal interface is likely to rapidly disintegrate. This is in contrast to other pregnancy complications, such as intrauterine growth retardation, which is associated with a comparatively small increase in programmed cell death among placental cells (0.14% versus 0.24%).³³ We suggest that apoptosis on the magnitude

we observed could have catastrophic consequences for pregnancy, such as the signs observed in preeclampsia.

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