Invasive cytotrophoblast apoptosis in pre-eclampsia

Olga Genbacev¹, Elaine DiFederico², Mike McMaster² and Susan J. Fisher¹,²,³,⁴,⁵

¹Department of Stomatology, ²Department of Obstetrics, Gynecology and Reproductive Sciences, ³Department of Pharmaceutical Chemistry and ⁴Department of Anatomy, University of California San Francisco, San Francisco, CA 94143, USA
⁵To whom correspondence should be addressed at: HSW 604, University of California San Francisco, San Francisco, CA 94143-0512, USA

Pre-eclampsia is a serious pregnancy complication diagnosed by signs of widespread maternal endothelial dysfunction. In normal pregnancy, a subpopulation of placental cytotrophoblast stem cells executes a differentiation programme 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. In pre-eclampsia, cytotrophoblasts fail to differentiate along the invasive pathway. The functional consequences of this abnormality negatively affect interstitial and endovascular invasion, thereby compromising blood flow to the maternal–fetal interface. To determine whether abnormal differentiation and/or hypoxia leads to apoptosis of invasive cytotrophoblasts, we used the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) 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 little or no apoptosis in any location, but in samples from patients with pre-eclampsia, 15–50% of the cytotrophoblast subpopulation that invaded the uterine wall was labelled (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 pre-eclampsia 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.

Key words: apoptosis/Bcl-2/cytotrophoblast/placenta/pre-eclampsia

Introduction
The pre-eclampsia syndrome affects ~7% of nulliparous women (Levine et al., 1997). The mother shows signs and symptoms, such as high blood pressure, proteinuria and oedema, that suggest widespread alterations in endothelial function (Roberts et al., 1989). 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 the mid-second trimester until term; hence the name pre-eclampsia, from the Greek eklampsis, meaning sudden flash or development.

Both the aetiology 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 develop this syndrome (Albrecht and Tomich, 1996). Pre-eclampsia also can occur in hydatidiform mole, a condition in which genetically abnormal placental tissue (e.g. trophoblast) proliferates in the absence of a fetus (Chun et al., 1964). In all cases the only known cure is removal of the placental tissue. If this is done before term, it can cause iatrogenic prematurity, further contributing to the morbidity and mortality associated with pre-eclampsia.

The placenta’s role in pre-eclampsia has been enigmatic. Microscopic analyses of placental speci-
mens 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 (Brosens et al., 1972; Gerretsen et al., 1981; Khong et al., 1986; Zhou et al., 1993). 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 a flow of maternal blood to the placenta. In pre-eclampsia, the interstitial component of invasion is variably compromised, with abnormally shallow invasion being most often associated with the appearance of signs in early gestation (Y.Zhou and S.J.Fisher, unpublished results). However, endovascular invasion is consistently rudimentary and, 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 pre-eclampsia. Our knowledge of the ability of the cells to intricately switch their adhesion molecule expression during the invasion process has been instrumental in 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 (e.g. E-cadherin, integrin α6β4), and they up-regulate the expression of those that are important for endothelial cell function (e.g. VE-cadherin, integrin αVEβ3, αVEβ1; Zhou et al., 1997b). Thus, during normal pregnancy, invasive cytotrophoblasts mimic the adhesion receptor phenotype of endothelial cells. We postulate that this unusual phenomenon plays an important role in the process whereby these cells form vascular connections with the uterine vessels. In pre-eclampsia, most aspects of this transition fail to occur, and undifferentiated, epithelial-like cytotrophoblast stem cells are found within the uterus (Zhou et al., 1997a). This failure ultimately limits the supply of maternal blood to the placenta and fetus, an effect thought to be closely linked to the pathophysiology of this disease. We used information about the morphological aspects of cytotrophoblast invasion in normal pregnancy and in pre-eclampsia to formulate hypotheses about the regulatory factors involved. In normal pregnancy cytotrophoblasts invade large-bore arterioles, where they are in contact with well-oxygenated maternal blood. In pre-eclampsia, however, invasive cytotrophoblasts are relatively hypoxic.

**In-vitro observations**

Reasoning that both observations might reflect the effects of oxygen on cytotrophoblast differentiation and invasion, we used an in-vitro model to test this hypothesis (Genbacev et al., 1996). When early gestation cytotrophoblast stem cells are cultured under standard conditions (20% O2), they differentiate and invade, replicating many aspects of the in-vivo process. Specifically, the cells proliferate at a low rate and rapidly invade extracellular matrix (ECM) substrates. This requires them to switch their repertoire of integrin cell-ECM receptors, which serve as stage-specific antigens marking specific transitions in the differentiation process. When we lowered oxygen tension to 2%, we found that many of the basic processes of the cells did not change. However, their incorporation of [3H]-thymidine and bromodeoxyuridine increased markedly. Moreover, they failed to invade ECM substrates, at least in part because of their inability to switch their integrin repertoire completely. These changes mimic many of the alterations in cytotrophoblast differentiation and invasion that occur in pre-eclampsia and suggest one possible mechanism by which a reduction in maternal blood flow to the placenta could contribute to the altered placental phenotype associated with pre-eclampsia.

**Observations on placental biopsies**

We recently tested the hypothesis that in pre-eclampsia the presence of abnormally differentiated fetal cytotrophoblasts among the resident maternal cells of the uterus could trigger apoptosis of one
or both populations. We studied tissue samples, obtained from nine pre-eclamptic and nine control patients, that contained floating villi as well as anchoring villi and the portion of the uterus to which they were attached (DiFederico et al., 1999). Apoptotic cells in tissue sections were detected by in-situ labelling of DNA strand breaks (TUNEL method), and cytotrophoblasts were identified by staining with anti-cytokeratin (Fisher et al., 1989).

In floating villi from either sample population, there was no evidence of apoptotic nuclei in trophoblast cells; a few stromal cells (≤1%) in the villous cores were randomly labelled (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 labelled. Other investigators have similarly detected relatively few apoptotic cells in the human placenta from the first trimester onward (Smith et al., 1997b).

A tissue sample with anchoring villi that was obtained from a patient diagnosed with severe pre-eclampsia at 26 weeks of gestation also contained numerous cytokeratin-positive cytotrophoblasts (Figure 1C). As we and others have previously described (Brosens et al., 1972; Zhou et al., 1993, 1997a), invasion was limited to the superficial portion of the uterus. TUNEL 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). Nonetheless, a few TUNEL-negative cells per field were labelled. Other investigators have similarly detected relatively few apoptotic cells in the human placenta from the first trimester onward (Smith et al., 1997b).

A tissue sample with anchoring villi that was obtained from a patient diagnosed with severe pre-eclampsia at 26 weeks of gestation also contained numerous cytokeratin-positive cytotrophoblasts (Figure 1C). As we and others have previously described (Brosens et al., 1972; Zhou et al., 1993, 1997a), invasion was limited to the superficial portion of the uterus. TUNEL 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. To identify these cells, we stained adjacent sections with antibodies that recognized either immune (anti-CD45) or decidual cells (anti-prolactin). The cytokeratin-negative cells that labelled 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 labelled 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 pre-eclamptic patients, only apoptotic cytotrophoblasts in the uterine wall were observed.

Despite the intense nuclear staining of invasive cytotrophoblasts in severe pre-eclampsia, many of the TUNEL-labelled cytotrophoblast nuclei had relatively normal sizes and shapes, suggesting they were in the initial stages of apoptosis (inset, Figure 1D). Somewhat fewer labelled cells had condensed, fragmented nuclei (Figure 1F). Hoechst staining demonstrated a similar spectrum of nuclear morphologies. While the nuclei of some cytokeratin-positive cells had a normal appearance, many others showed evidence of either chromatin condensation or fragmentation (data not shown). Previous reports suggest that apoptotic cells are recognized, ingested, and degraded beyond histological recognition in 1–2 h (Kerr et al., 1972; Coles et al., 1993). Thus, our data likely suggest that pre-eclampsia is associated with the sudden onset of widespread apoptosis of invasive cytotrophoblasts and, sometimes, of maternal (decidual) cells within the uterine wall.

The numbers of apoptotic (TUNEL-labelled) invasive cytotrophoblasts that were detected in tissue samples obtained from control (n = 9) and pre-eclamptic (n = 9) patients at 26–40 weeks of gestation are summarized in Figure 2. Little or no apoptosis was observed in the control samples until term, when most of the invasive cytotrophoblasts with labelled nuclei were localized in the deeper portions of the decidua (data not shown). In contrast, substantial apoptosis (mean 30.1 ± 17.9% of cytotrophoblasts) was observed in all but one of the pre-eclampsia samples (P = 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.

Next, we used sections cut from these same tissue samples to determine whether pre-eclampsia

Pre-eclampsia: invasive cytotrophoblast apoptosis

...
Pre-eclampsia is associated with a large increase in terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) of cytotrophoblasts (CTB) within the uterine wall. Sections of placental bed biopsy specimens were from the following patients: (A, B) control at 26 weeks of gestation (26 W, CON); (C, D) patient with severe pre-eclampsia at 26 weeks of gestation (26 W, SPE); (E, F) patient with severe pre-eclampsia at 31 weeks of gestation (31 W, SPE). Apoptotic cells (B, D, F) were identified by the TUNEL method, a commercial kit that fluorescence-labels DNA strand breaks (Boehringer-Mannheim, Indianapolis, IN, USA). To identify trophoblasts among other fetal and maternal cells, TUNEL-stained frozen sections were double stained with a rat monoclonal antibody (Fisher et al., 1989) that specifically reacts with cytokeratin (A, C, E). Antibody binding was detected by using a rhodamine-conjugated secondary antibody as previously described. In cells labelled with TUNEL, the nuclei often appeared to be relatively intact (D, inset), although clusters of cells with fragmented nuclei were also seen (F, arrows). In some specimens, apoptosis was observed in cells that did not express cytokeratin (A, C, E). Staining of adjacent sections with anti-CD45 showed that the smaller labelled 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. (Reprinted from DiFederico et al., 1999, with permission of American Journal of Pathology.)
Pre-eclampsia: invasive cytotrophoblast apoptosis

Figure 2. Percentage of terminal deoxynucleotidyl transferase-mediated dUTP nick end labelled (TUNEL) invasive cytotrophoblasts in tissue samples obtained from control (n = 9) and pre-eclamptic (n = 9) patients at 26-40 weeks of gestation. Each data point corresponds to the percentage of cytokeratin-positive cytotrophoblasts (mean ± SEM) within the uterine wall that were labelled with TUNEL. Percentages were calculated by examining 5-7 sections from at least three separate tissue blocks. (Reprinted from DiFederico et al., 1999 with permission of American Journal of Pathology.)

is associated with a change in expression of Bcl-2, an oncoprotein that can suppress programmed cell death in both normoxic and hypoxic conditions (Jacobson and Raff, 1995; Shimizu et al., 1995).

We first used an antibody to Bcl-2 to stain sections of floating villi found in the placenta proper. As has been shown by other investigators (Lea et al., 1997; Uckan et al., 1997; Marzioni et al., 1998), 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. This pattern did not change when the placental sample was obtained from a pregnancy complicated by pre-eclampsia. We then examined Bcl-2 expression by invasive cytotrophoblasts that were found within the uterine wall, i.e. the population in which a significant number of cells in pre-eclamptic samples were undergoing programmed cell death. In control pregnancies, groups of cytotrophoblasts stained intensely; a few cytokeratin-positive cells (≤20%) did not react with the anti-Bcl-2 antibody (Figure 3A and B). In contrast, no staining above background was detected in cytotrophoblasts that invaded the uteri of patients with pre-eclampsia (e.g. Figure 3C and D).

The role of apoptosis in the pre-eclamptic uterus

Our finding that fetal cytotrophoblasts within the uterine walls of pre-eclamptic patients undergo programmed cell death fits into the current paradigm of how apoptosis functions in vivo. Namely, one important purpose of apoptosis is the selective deletion of cells that are abnormally differentiated and consequently functionally impaired (reviewed by Ashkenas and Werb, 1996). The presence of apoptotic cells has been reported in the placenta and decidua from normal (Smith et al., 1997b; Huppertz et al., 1998) and abnormal (Smith et al., 1997a; Kokawa et al., 1998a,b) pregnancies. As our previous work has shown, in pre-eclampsia the repertoire of adhesion molecules expressed by invasive cytotrophoblasts is significantly altered from that observed in normal pregnancy (Zhou et al., 1993, 1997a), suggesting that the cells’ interactions with maternal cells and uterine extracellular matrix molecules are also abnormal. As
Figure 3. The anti-Bcl-2 staining pattern of invasive cytotrophoblasts (CTB) is selectively reduced in pre-eclampsia. Sections immediately adjacent to those used for the detection of apoptotic cells were double stained (2 h) with a mixture of mouse anti-Bcl-2 (5 μg/ml; Oncogene Research Products, Cambridge, MA, USA) and rat anti-cytokeratin. Antibody binding was detected and samples were examined as described above. (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 pre-eclampsia (D) failed to react with this antibody, suggesting greatly reduced Bcl-2 expression. (Reprinted from DiFederico et al., 1999 with permission of American Journal of Pathology.)

has been observed in other systems (Hermiston and Gordon, 1995; Frisch and Ruoslahti, 1997), such anomalies can transmit intracellular signals that lead to apoptosis rather than survival.

Since many apoptotic cells appear to enter the cell cycle (Franceschi, 1989; Meikrantz and Schlegel, 1995; White, 1996), the unusual effects of low oxygen on cytotrophoblast mitotic activity could also be relevant to our finding that these cells undergo apoptosis in pre-eclampsia. 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. In our in-vitro experiments in which we cultured villous explants on an extracellular matrix in a hypoxic environment (2% O₂), cytotrophoblasts continued to progress through the cell cycle while they differentiated, albeit abnormally (Genbacev et al., 1997).

A normal mitogenic response results in the passage of cells from G₀ to G₁. Having entered G₁, a cell has three possible fates: (i) normal progression through the cell cycle, which results in division; (ii) G₁ arrest; or (iii) apoptosis. We postulate that prolonged exposure to a hypoxic environment, the likely scenario in pre-eclampsia, could eventually lead abnormally differentiated and functionally impaired cytotrophoblasts at the maternal–fetal interface to exit the cell cycle in G₁ and to progress toward apoptosis.

Our findings could help explain several well-recognized clinical aspects of this syndrome. For example, pre-eclampsia is associated with fibrin deposition at the maternal–fetal interface (Kanfer et al., 1996). Recent data suggest that phosphatidylserine, a neo-antigen on the surface of apoptotic cells, has potent procoagulant activity (Casciola-Rosen et al., 1996). Thus, it seems likely that cytotrophoblasts undergoing programmed cell death could elicit fibrin deposition, as well as platelet activation, another common feature of pre-eclampsia (Redman, 1990). Whether or not this phenomenon is also relevant to the fact that women with antiphospholipid antibodies have an increased risk of developing pre-eclampsia remains to be determined (Welsch and Branch, 1997).

Another unique aspect of the clinical presentation of pre-eclampsia is its sudden appearance, particularly in patients with the most severe signs. The finding that many of the fetal cytotrophoblasts in direct contact with resident uterine cells are undergoing programmed cell death suggests that the maternal–fetal interface is rapidly disintegrating. 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%; Smith et al., 1997a). We suggest that apoptosis of the magnitude we observed could have catastrophic consequences for
pregnancy, consequences like the signs observed in pre-eclampsia.

Acknowledgements

This work was supported by a grant from the National Institutes of Health (HD 30367). The authors thank Ms. Rebecca Joslin for excellent technical assistance and Ms. Evangeline Leash for excellent editorial assistance.

References


Pre-eclampsia: invasive cytotrophoblast apoptosis


