Phagocytosis of Necrotic but Not Apoptotic Trophoblasts Induces Endothelial Cell Activation

Qi Chen, Peter R. Stone, Lesley M.E. McCowan, Larry W. Chamley

Abstract—It is hypothesized that preeclampsia is caused by factors from the placenta that induce endothelial cell activation. Trophoblasts are cells that may be shed from the placenta, then deported in the maternal blood, and finally become trapped in the pulmonary capillaries. The ultimate fate of deported trophoblasts is unknown, but to prevent clogging of the pulmonary circulation they must be cleared from the capillary beds. We examined the hypothesis that endothelial cells phagocytose deported trophoblasts and also examined the consequent effects of the trophoblasts on endothelial cells. Fluorescently labeled trophoblast–derived choriocarcinoma cells were induced to become apoptotic or necrotic and exposed to endothelial cell monolayers. Inhibiting the phosphatidylinositol 3-kinase and p38 mitogen-activated protein kinase pathways blocked both expression of ICAM-1 and phagocytosis, whereas inhibition of the P42/44 mitogen-activated protein kinase pathway blocked only ICAM-1 expression. This work suggests that endothelial cells can phagocytose deported trophoblasts and that the mechanism of trophoblast death (apoptotic or necrotic) could have major effects on the maternal vascular response to shed trophoblasts. (Hypertension. 2006;47:116-121.)

Key Words: endothelium □ cell adhesion molecules □ hypertension, pregnancy □ leukocytes

Preeclampsia (PE) is characterized by maternal vasoconstriction, multiorgan dysfunction, and hypertension and appears to be caused by a factor or factors released from the placenta, because this disease only occurs when placental tissue is present. The vascular endothelial cells of women affected by PE become activated, and this activation (as opposed to mechanical injury) of endothelial cells is thought to contribute significantly to the pathogenesis of PE. During pregnancy, the human placenta is bathed in maternal blood. Two layers of placental cells, multinucleated syncytiotrophoblast, separate the remainder of the fetoplacental unit from the maternal blood. These 2 trophoblast populations are epithelial cells, which have a limited life span. Like other epithelia, for example, in the gut, aged and dead trophoblasts are shed from the placenta at the end of their life cycle. When trophoblasts are shed, they enter the maternal blood that is bathing the placenta and are then transported via the draining uterine veins into the maternal circulation. This process is called trophoblast deportation and was first described in 1893 by Schmorl, who reported syncytiotrophoblast lodged in the lungs of women who had died of eclampsia. Subsequently, it has been shown that trophoblast deportation is a feature of normal pregnancy, which may be more pronounced in PE. This has led to the hypothesis that shed/deported trophoblasts may be the placental factor that induce the generalized endothelial cell dysfunction seen in PE. It has been estimated that 150 000 trophoblasts are deported from the placenta daily in normal pregnancy. Many of these deported trophoblasts become trapped in the capillary beds of the lungs, but clogging of the maternal lungs by trophoblasts does not occur in normal pregnancy. That the maternal capillary beds do not become clogged with trophoblasts suggests that a mechanism must exist to clear these shed cells from the maternal lungs and circulation. However, at present, we do not know what this mechanism is. It is likely that professional phagocytes of the maternal immune system, such as macrophages, play a significant role in clearing deported trophoblasts, but recently it has been shown that other cell types that are considered nonprofessional phagocytes are also important in clearing dead cells, especially apoptotic cells, from tissues. Such nonprofessional phagocytes include endothelial cells. However, it is not known whether endothelial cells are capable of phagocytosing deported trophoblasts. Therefore, we undertook this study to examine whether endothelial cells could phagocytose dead trophoblasts and the consequences that this would have on endothelial cell biology.

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Methods

Ethics
This study complies with the requirements of the institutional ethics committee.

Cell Culture
All of the culture media and FBS were purchased from Invitrogen. The human microvascular endothelial cell line (HMEC-1) was obtained from the National Centre for Infectious Diseases and grown in MCDB 131 as described previously. The trophoblast-derived chorioarci nella cell lines Jar and Jeg-3 were used to model trophoblasts and were grown in DMEM/F12 as described previously. U937 cells, a monocyte/macrophage-like cell line, were grown in DMEM/F12 containing 10% FBS and 1% penicillin/streptomycin.

Induction of Cell Death
Before inducing cell death, Jeg-3 and Jar cells were labeled with 1 µmol/L Red Fluorescent Cell Tracker Stain [5-chloromethoxy carboxysemianthrodifulor-1 (SNARF-1); Molecular Probes] by incubation at 37°C for 2 hours. Jeg-3 and Jar cells were induced to undergo apoptotic death by exposure to UV light (30W) for 1 hour. Apoptotic death was confirmed by immunohistochemical examination for the cytokeratin neoepitope M30, which is produced by cleavage of cytokeratin by caspases and is, thus, a marker of the later stages of apoptosis. The treated cultures contained cells that were 50% to 70% positive for the M30 neoepitope.

Jeg-3 and Jar cells were induced to undergo necrotic death by a freeze–thaw cycle as described previously. Briefly, cells were frozen to −20°C for 1.5 hours and then slowly thawed at room temperature. To measure the proportion of necrotic trophoblasts, cells were incubated with YO-PRO-1 and propidium iodide from a commercial cell death analysis kit (Vibrant apoptosis kit number 7; Molecular Probes) for 30 minutes on ice, and then the stained cells were analyzed by flow cytometry (FACScan, BD Biosciences). Ninety percent of the cells were necrotic.

Endothelial Cell Activation Assay
HMEC-1 cells (10⁶) were seeded into each well of 96-well sterile culture plates and grown to confluence, and then a total of 4000 apoptotic, necrotic, or, for the experiments described in Figure 2C, various mixtures of apoptotic and necrotic trophoblasts per well were added, and the cells were incubated at 37°C for 24 hours. To determine whether HMEC-1 became activated after exposure to dead trophoblasts, a cell-based ELISA was used to quantify the expression of ICAM-1, E-selectin, or vascular cell adhesion molecule (VCAM), as described previously. In experiments involving pharmacological inhibitors, the inhibitors PD98059 (10 µmol/L), SB203580 (10 µmol/L), LY294002 (10 µmol/L), or cyclochalasin B (10 µg/mL; Sigma) were added to the endothelial cell cultures for 60 minutes before the addition of the dead trophoblasts, and the inhibitors were maintained in the cultures along with the trophoblasts.

Detection of Phagocytosis
HMEC-1 cells (2.5 × 10⁵) were grown on plastic microscope slide coverslips (Biolab) in 6-well plates until confluent and were then labeled with 1 µmol/L Green Cell Tracker stain, 5-chloromethyl-7-hydroxyxoumarin (Molecular Probes), for 2 hours at 37°C and 5% CO₂. SNARF-1-labeled apoptotic or necrotic trophoblasts (6.25 × 10⁵ per well) were then incubated with the confluent monolayers of endothelial cells for 24 hours at 37°C Controls cultures of HMEC-1 monolayers were cultured in the absence of trophoblasts. The cultures were washed, fixed with 4% paraformaldehyde, and the coverslips were mounted onto glass microscope slides with Citiflour (Aqar Scientific). The cells were then examined by confocal microscopy using a Leica model TCS SP2 confocal microscope.

For experiments to quantify the effects of pharmacological inhibitors, 10⁶ HMEC-1 cells were seeded and grown to confluence in 12-well culture plates, and the inhibitors PD98059 (10 µmol/L), LY294002 (10 µmol/L), SB203580 (10 µmol/L), or cyclochalasin B (10 µg/mL) were added 60 minutes before, as well as during, incubation for 24 hours at 37°C with either apoptotic or necrotic trophoblasts. The wells were then washed and the HMEC-1 cells harvested by trypsinization before quantification of phagocytosis by 2-color flow cytometry. The percentage of HMEC-1 cells containing trophoblast-derived fluorescence was determined and compared with control cultures in which the inhibitors were omitted.

U937 Adhesion Assay
HMEC-1 cells were grown to confluence in 12-well culture plates and then exposed to 8 × 10⁵ apoptotic or necrotic trophoblasts per well for 24 hours. The cultures were washed, 10⁶ U937 cells, which had been labeled with SNARF-1, were added, and the incubation continued for 3 additional hours at 37°C. The cultures were then washed to remove all of the nonadherent U937 cells. The cultures were then trypsinized to release the adherent U937 cells, which were quantified by flow cytometry and expressed as a percentage of the baseline adhesion of U937 cells to control cultures of untreated HMEC-1 cells.

Statistical Analysis
For endothelial cell activation ELISAs, the effects of all of the samples were measured in duplicate wells, and each experiment was repeated three times. All of the other experiments were repeated 3 times. Data are presented as mean ± SD. The statistical significance of the results was assessed by t test or ANOVA as appropriate.

Results

Endothelial Cells Phagocytose Both Apoptotic and Necrotic Trophoblasts
To examine whether endothelial cells could phagocytose shed trophoblasts, we labeled the trophoblast-derived cell lines Jar and Jeg-3 with red fluorescent cell tracker stain, and then induced either apoptotic or necrotic death of the cells. The fluorescent dead Jar and Jeg-3 cells were then exposed for 24 hours to monolayers of HMEC-1 endothelial cells that had been labeled with green fluorescent cell tracker stain. Examination by confocal microscopy revealed that many of the HMEC-1 cells contained trophoblast-derived red fluorescent particles (Figure 1). Serial images taken in the z-plane confirmed that the trophoblast-derived material was contained within the endothelial cells and not attached to the outside of the cells (see supplementary data, available online at http://www.hypertensionaha.org).

Endothelial Cells Are Activated by Necrotic but Not by Apoptotic Trophoblasts
To investigate whether phagocytosis of apoptotic or necrotic trophoblasts had differential effects on endothelial cells, the expression of adhesion molecules was measured on endothelial cells that had been exposed to either apoptotic or necrotic trophoblasts. The levels of E-selectin and VCAM increased significantly after endothelial cells were treated with necrotic but not apoptotic trophoblasts (P < 0.05; Figure 2A). This response was similar when both necrotic Jar or Jeg-3 cells were used and the level of ICAM-1 expression on endothelial cells after exposure to necrotic trophoblasts was dose dependent (Figure 2B; P < 0.05). To additionally investigate the effects of dead trophoblasts on endothelial cells, we conducted mixing experiments in which HMEC-1 cells were treated with mixtures containing different ratios of
Phagocytosis Is Required for the Expression of ICAM-1 in Endothelial Cells

To confirm that increased expression of ICAM-1 by endothelial cells was dependent on phagocytosis of the necrotic trophoblasts, we quantified the expression of ICAM-1 by endothelial cell monolayers that had been exposed to necrotic trophoblasts in the presence (or absence) of the phagocytosis inhibitor cytochalasin B. Cytochalasin B treatment reduced the percentage of HMEC-1 cells that had phagocytosed necrotic Jar cells by 45% and Jeg-3 cells by 50% (P<0.05) and significantly inhibited the increase in ICAM-1 expression induced by phagocytosis of the necrotic trophoblasts (P<0.05; Figure 3).

Phagocytosis of Necrotic Trophoblasts by Endothelial Cells Increases the Adhesion of U937 Cells

To investigate whether the increased expression of ICAM-1 by endothelial cells after treatment with necrotic trophoblasts could increase the adhesion of leukocytes, we used the monocyctic cell line U937. Endothelial cell monolayers were exposed to necrotic or apoptotic trophoblasts, and then the adhesion of fluorescence labeled U937 cells was quantified by flow cytometry. Significantly more U937 cells (P<0.05; Figure 4) adhered to endothelial cells that had been treated with necrotic trophoblasts than to control endothelial cells. In contrast, there was no increase in the adhesion of U937 cells to endothelial cell monolayers that had been exposed to apoptotic trophoblasts.

Effect of PI3K and MAPK on Phagocytosis in Endothelial Cells

Because kinases are known to be involved in many cellular pathways, including phagocytosis, we wished to investigate whether PI3K and MAPK were preventing the phagocytosis of necrotic trophoblasts or were acting downstream in the direct regulation of ICAM-1 expression. The PI3K inhibitor LY294002 and the p38 MAPK inhibitor (SB203580) significantly reduced (P<0.05) the number of endothelial cells that phagocytosed necrotic trophoblasts (Figure 6). However, the p42/44 MAPK inhibitor did not significantly alter the phagocytosis of necrotic trophoblasts, suggesting that PI3K and p38 MAPK are involved in regulating the phagocytosis of necrotic trophoblasts, whereas the p42/44 MAPK is involved in the direct regulation of ICAM expression subsequent to phagocytosis of necrotic trophoblasts.

Discussion

During normal pregnancy, large numbers of multinucleated fragments of syncytiotrophoblast, called syncytial knots, are shed daily into the maternal circulation. These syncytial knots are too large to pass through the pulmonary capillary beds and are trapped in the maternal lungs.7,8,17 Because the lungs of most women do not become clogged by shed trophoblasts, there must be a mechanism for clearing these shed trophoblasts.18 Because trophoblasts trapped in the maternal lungs are likely to be in close apposition to the pulmonary vascular endothelial cells, we hypothesized that maternal endothelial cells might phagocytose dead trophoblasts. Our results show that this is indeed the case and that endothelial cells can phagocytose dead trophoblasts regardless of whether the trophoblasts have died by a necrotic or an apoptotic mechanism. However, phagocytosis of necrotic and apoptotic trophoblasts has quite different consequences for the endothelial cells. Whereas phagocytosis of apoptotic trophoblasts did not induce markers of endothelial cell activation, phagocytosis of necrotic trophoblasts induced increased expression of the adhesion molecule ICAM-1 (CD54), but the expression of VCAM and E-selectin was not affected by phagocytosis of either apoptotic or necrotic trophoblasts. Others have shown that the sera of women with PE contains increased amounts of soluble adhesion molecules including ICAM, although there is some inconsistency in these studies regarding exactly which soluble adhesion molecules are elevated.19–21 Our
work indicates that phagocytosis of necrotic trophoblasts leads to increased expression of ICAM on the endothelial cell surface, thus increasing the pool of endothelial ICAM from which the soluble form of the protein may be derived.

It is possible that simple cell–cell contact between the necrotic trophoblasts and the endothelial cells is sufficient to induce increased expression of ICAM-1 by the endothelial cells. That phagocytosis of the necrotic trophoblasts was essential in the upregulation of ICAM-1 by endothelial cells was demonstrated by the finding that the increase in ICAM-1 expression was abolished by treating endothelial cells with the phagocytosis inhibitor cytochalasin B. This treatment...
inhibited both the phagocytosis of dead trophoblasts and the increase in ICAM-1 expression. Others have shown previously that microvillous particles shed from the membranes of syncytiotrophoblasts can induce endothelial cell dysfunction and have suggested that they may be important in the pathogenesis of PE because their small size allows them to freely pass through the maternal lungs and enter the peripheral circulation.1,22 Our work demonstrates that endothelial cells are also capable of phagocytosing entire (dead) trophoblasts, which could lead to the endothelial dysfunction that is characteristic of PE.

Our results also demonstrate that the effects induced by phagocytosing necrotic trophoblasts are dose dependent, and the more necrotic trophoblasts that endothelial cells are exposed to the greater the effect on endothelial cell activation. Importantly, based on our dose-response experiments (Figure 2B), as few as \( \approx 60 \) or 120 (Jeg-3 or Jar, respectively) necrotic trophoblasts per mm\(^2\) of endothelial cells was sufficient to induce detectable endothelial cell activation in our system. Although it has been estimated that 150 000 trophoblasts are shed daily in normal pregnancy, it is unknown what proportion of these are necrotic, but our data suggest that relatively few necrotic trophoblasts may be required to commence the process of endothelial cell activation. We were able to demonstrate that when a mixture of apoptotic and necrotic trophoblasts was exposed to endothelial cells, the apoptotic trophoblasts did not protect the endothelial cells from activation. These results suggest that in vivo shedding of even relatively small numbers of necrotic trophoblasts could potentially lead to the activation of pockets of endothelial cells in the pulmonary vessels and in other capillary beds where trophoblasts become trapped and phagocytosed. We have not as yet investigated whether these activated endothelial cells secrete products that could promote the spread of endothelial cell activation to other sites. However, we did demonstrate that the increased expression of ICAM-1 (and potentially other adhesion molecules that we did not study) by endothelial cells following phagocytosis of necrotic trophoblasts increased the propensity of U937 monocytes to adhere to the endothelial cells. This finding confirms that the increased level of ICAM-1 expression had a biological effect and also raises the possibility that this interaction between the activated endothelial cells and monocytes could lead to the monocytes producing a soluble product, which could result in more widespread endothelial cell activation.

We have shown that the expression of ICAM-1 by endothelial cells treated with necrotic trophoblasts can be abolished by inhibitors of the PI3K, p38 MAPK, sand p42/44 MAPK pathways, but that inhibition of the PI3K and p38 MAPK pathways also abolished the phagocytosis of the trophoblasts. In contrast, inhibiting the p42/44 MAPK pathway did not affect the phagocytosis of trophoblasts by endothelial cells. The p38 MAPK pathway is known to be involved in regulating both phagocytosis and ICAM-1 expression.23–25 Likewise, the PI3K pathway is known to be involved in phagocytosis by immune cells.26 Our work demonstrates that the PI3K pathway is also important in phagocytosis by endothelial cells. Whether the PI3K pathway is also involved in directly regulating the increased expression of ICAM after the phagocytosis of necrotic trophoblast cannot be determined from our work. However, it is clear that the p42/44 MAPK pathway regulates the increased expression of ICAM-1 without affecting phagocytosis. Manipulation of this pathway may be a potential target for preventing some of the effects of endothelial cell activation. It has been shown previously that the p42/44 MAPK pathway is involved in the upregulation of ICAM-1 expression by endothelial and epithelial cells in response to various stimuli including inflammatory cytokines and infection by *Chlamydia pneumoniae*.27–29 However, it is unclear how necrotic trophoblasts activated this pathway to induce the expression of ICAM-1 in our system.

It has been suggested previously that increased necrotic trophoblast shedding occurs in PE in response to the poor perfusion of the placenta.30 We believe our results are the first demonstration that a consequence of this increased shedding of necrotic trophoblasts could be the phagocytosis of the

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**Figure 5.** The expression of ICAM-1 by HMEC-1 monolayers exposed to necrotic Jar or Jeg-3 cells in the presence or absence of inhibitors of p42/44 MAPK (PD98059; 10 μmol/L), PI3K (LY294002; 10 μmol/L), or p38 MAPK (SB203580; 10 μmol/L) was determined by cell-based ELISA. Experiments were conducted in duplicate on 3 separate occasions. Bars=SD. *P<0.05.

**Figure 6.** The involvement of the PI3K, as well as the p38 and p42/44 MAPK pathways in the phagocytosis of necrotic trophoblasts by endothelial cells was investigated by exposing HMEC-1 monolayers to red fluorescent-labeled necrotic Jar or Jeg-3 cells in the presence of the inhibitors PD98059 (10 μmol/L; p42/44 MAPK), LY294002 (10 μmol/L; PI3K), or SB203580 (10 μmol/L; p38 MAPK). The percentage of HMEC-1 cells containing trophoblast-derived fluorescence was compared with uninhibited cultures by 2-color flow cytometry. *P<0.05.
necrotic trophoblasts by endothelial cells, thereby directly activating the endothelial cells. Because the activation of endothelial cells is dependent on a sufficient dose of necrotic trophoblasts, such activation would only occur in vivo when a critical threshold of necrotic trophoblast built up in the maternal circulation. Thus, a small amount of necrotic trophoblast shedding might be tolerated without generating an inflammatory response, but an increase in the quantity of necrotic trophoblast shed from the placenta would induce endothelial cell activation as is postulated to occur in PE.30

Perspectives
Here we have shown for the first time that endothelial cells can phagocytose dead trophoblasts and that the phagocytosis of necrotic trophoblasts causes endothelial activation. Although it has been known for many years that increased shedding of dead (probably necrotic) trophoblasts occurs in PE, it has been difficult to understand how this could contribute to the pathogenesis of PE, because most shed trophoblasts are trapped in the maternal lungs and do not reach the peripheral circulation. Our observation that endothelial cells can phagocytose dead trophoblast helps to explain, first, how the large numbers of trophoblast shed from the placenta are rapidly cleared from the lungs in normal pregnancy to prevent clogging of the maternal pulmonary circulation, and, second, the activation of the endothelial cells after phagocytosis of necrotic trophoblasts suggests a mechanism by which the endometrium can become activated. Importantly, our finding that inhibiting the p42/44 MAPK pathway blocks endothelial cell activation by necrotic trophoblasts but not the phagocytosis of the trophoblasts suggests that it may be possible to manipulate this pathway in vivo to allow the continued clearance of dead trophoblasts from the maternal circulation but prevent the subsequent activation of endothelial cells induced by phagocytosis of necrotic trophoblasts.

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References
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I. Confocal z-stack of an endothelial cell phagocytosing trophoblast-derived material