An *in vitro* model of human placental trophoblast deportation/shedding

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Deportation of trophoblast shed from the placenta into the maternal circulation was first described over 100 years ago. Despite this, little is known about the quantity or nature of the shed and deported trophoblasts. Neither do we have a clear understanding of the fate of deported trophoblasts nor do we have a clear understanding of their effects on the maternal physiology. This deficiency is largely due to the inaccessibility of deported trophoblasts *in vivo*. This study aimed to produce a model that would allow us to study deported trophoblasts. We devised a system for culturing placental explants of 12-week gestation in cell culture inserts with a stainless steel mesh bottom that allowed the ready harvesting of shed/deported trophoblasts. Immunohistochemical and morphologic investigations demonstrated that these *in vitro* shed/deported trophoblasts are similar to those found *in vivo* and that apoptotic, necrotic and viable trophoblasts were shed from the explants. Inhibiting caspases induced a change from predominantly apoptotic to predominantly necrotic trophoblast shedding. We have devised an *in vitro* model that allows the collection of shed/deported trophoblasts which will significantly enhance our ability to study these cells. Our preliminary investigations confirm that apoptosis plays an important role in trophoblast shedding/deportation.

**Key words**: apoptosis/deportation/explant/model/trophoblast

**Introduction**

Trophoblast deportation is the process of transporting trophoblasts that are shed from the placenta to distal sites via the maternal blood. This process was first reported over 100 years ago by Schmorl (1893) who described syncytiotrophoblast-like fragments trapped in the lungs of women who had died of eclampsia. Subsequently, it has been shown that trophoblast deportation is a physiological feature of normal pregnancy that may be exacerbated in pre-eclampsia, eclampsia and other diseases of pregnancy (Fox, 1965; Alvarez *et al.*, 1967; Boyd and Hamilton, 1970; Chua *et al.*, 1991; Johansen *et al.*, 1999).

Several reports have suggested that programmed cell death (apoptosis) may initiate degeneration in the syncytiotrophoblast this layer naturally ages, leading to shedding of multinucleated fragments of the syncytiotrophoblast called syncytiial knots into the maternal circulation (Nelson, 1996; Huppertz *et al.*, 1998; Marzioni *et al.*, 1998; Chan *et al.*, 1999; Mayhew *et al.*, 1999). Syncytiotrophoblast has also been noted to have necrotic features *in vivo* and *in vitro* (MacLennan *et al.*, 1972; Kaufmann, 1985; Palmer *et al.*, 1997), and it has been suggested that necrotic trophoblasts may also be shed from the placenta, and this might be especially the case in pathological pregnancies (Huppertz and Kingdom, 2004).

Although several causes for trophoblast shedding have been proposed, currently it is thought that most trophoblast shedding is simply the result of the loss of aged or damaged trophoblasts in a process which is analogous to the shedding of other epithelia, such as occurring in the gut (Zhao *et al.*, 2004). There is relatively little direct experimental evidence to support this, and estimates of the quantity of trophoblast shed in normal and diseased pregnancies vary widely. Thus, there is a large deficit in our knowledge about the nature and quantity of trophoblasts that are shed and deported during pregnancy, and we understand little about the mechanisms that lead to this shedding. This deficit is largely due to the inaccessibility of deported trophoblasts that are by and large thought to be trapped in the maternal lungs and also due to the inaccessibility of the placental site during pregnancy. Here, we report the development and characterization of a model, based on placental villous explant cultures, that allows us to harvest sufficient quantities of shed trophoblasts to investigate their nature, origins and potentially their effects on maternal physiology.

**Materials and methods**

**Ethics of experimentation**

This study was approved by the regional Ethics Committee, and all placental tissues were obtained with informed consent.

**Placenta**

Placentae of 12-week gestations were obtained following elective surgical termination of pregnancy (TOP). The gestational age and fetal viability of all pregnancies before TOP were confirmed by ultrasound measurement of crown–rump length and fetal cardiac activity.

**Harvesting trophoblasts shed from placental explants *in vitro***

Placental tissue from 16 placentae was washed with ice-cold phosphate-buffered saline (PBS), dissected and rewashed with ice-cold PBS, and triplicate explants (individual explant weight was ∼40 mg) were transferred into Net-well™ inserts (400-μm mesh) in 12-well culture plates with 3 ml of Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium containing 10% fetal bovine serum (FBS), 5 ng/ml of epidermal growth factor, 5 μg/ml of insulin, 10 μg/ml of transferin, 100 μg/ml of l-glutamate, 20 nm sodium selenite, 5 ng/ml of hydrocortisone, 5 μg/ml of dexamethasone, 2.5 μg/ml of progesterone, 1 μg/ml of sodium selenite and 100 μg/ml of transferrin.
The effects of inhibiting caspases on trophoblast shedding

Triplicate explants from 12 placentae were cultured in the presence and absence of 5 μm caspase inhibitor (DEVD-CHO; Apopain inhibitor; #235423; Calbiochem) and harvested as described above. Trophoblasts shed from the explants were characterized and quantified by immunocytochemical staining.

Immunohistochemistry staining

Slides with the air-dried cells were thawed for 5 min at room temperature and were encircled with a Dako pen (DAKO, MedBio, Christchurch, New Zealand). Non-specific binding was blocked by incubation with 10% normal goat serum in PBS–Tween for 10 min at room temperature. After three washes with PBS–Tween, the slides were incubated with primary antibodies and with irrelevant, species or class-matched, control antibodies diluted in 10% normal goat serum in PBS–Tween as summarized in Table I for 1 h at room temperature. The slides with the air-dried cells were thawed for 5 min at 20°C. Shed trophoblasts were resuspended in 210 μl of PBS; then 30-μl aliquots were air-dried onto microscope slides and fixed with cold acetone (–20°C) for 10 min and then left to air-dry for 1 h. Slides with acetone-fixed air-dried trophoblasts were stored at –20°C until used in immunocytochemical staining. Cells shed from the explants were characterized by immunocytochemical staining.

Depletion of CD45-positive cells from cell suspensions

Shed cells that were harvested from the medium of explant cultures were resuspended in 0.1% FBS/PBS and incubated with pre-washed Dynabeads® Pan mouse IgG (Dynal Biotech Pty Ltd, Australia and New Zealand) (washing performed according to manufacturers’ instructions) for 30 min at 4°C with gentle rotation using a blood tube rotator. CD45-positive cells were harvested by applying the tube containing the cells to a Dynal MPC®-2 Magnetic Particle Concentrator for 3 min at room temperature. The supernatant, depleted of most contaminating CD45-positive cells, was aspirated into a fresh tube, and then the magnetic harvesting step was repeated twice to ensure all contaminating CD45-positive cells were removed. The final supernatant, containing shed trophoblasts depleted of CD45-positive cells, was washed twice with 0.1% FBS/PBS, and the trophoblasts were harvested by centrifugation at 300 × g for 8 min at 4°C and resuspended in 1 ml of DMEM/F12 medium supplemented with 10% FBS, 100 μg/ml streptomycin, 100 U/ml penicillin and 100 μg/ml l-glutamate, pH 7.4.

Determination of the viability of shed trophoblasts with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Shed trophoblasts that had been depleted of CD45-positive cells were incubated with 5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in DMEM/F12 medium supplemented with 10% FBS, 100 μg/ml streptomycin, 100 U/ml penicillin and 100 μg/ml of l-glutamate for 1 h at 37°C. The cells were washed, fixed with 4% (w/v) paraformaldehyde, air-dried and visualized immediately by light microscopy using a Nikon Eclipse E400 microscope. The numbers of MTT-positive cells were counted in the same manner as above. The shed trophoblasts were then further analysed by immunocytochemical staining with antibodies reactive with the M30 cytokeratin neoepitope (Roche Diagnostics, Auckland, New Zealand) and activated caspase-3 (Sigma, Australia). Shed trophoblasts were also stained with irrelevant, species or class-matched, control antibodies. The numbers of trophoblasts shed from the explants were quantified as above.

Statistical analysis

Data were analysed using the Student’s t-test or analysis of variance (ANOVA) as appropriate. These analyses were performed using Microsoft Excel (Office 97 software). Results were considered to be statistically significant if P < 0.05.

Results

An in vitro model of trophoblast deportation/shedding

Villus explants of ~40-mg wet weight were cultured in Net-well inserts, and the cells shed from the explants every 24 h were analysed by light microscopy and immunocytochemistry, revealing that leukocytes, red blood cells (RBCs) and two distinct types of trophoblasts, mononuclear trophoblasts and multinucleated syncytiotrophoblast fragments that we will refer to as syncytial knots, were shed (Figures 1 and 2A–D). The mononuclear trophoblasts varied in size from 7 to 34 μm in width (mean 15.16 ± 0.69) and from 9 to 54 μm in length (mean 23.39 ± 1.02). The syncytial knots varied greatly in size ranging from 16 to 171 μm in width (mean 43.11 ± 2.75) and from 23 to 366 μm in length (76.25 ± 6.66) and contained up to several hundred nuclei per syncytiotrophoblast. The nuclei were usually small and contained densely packed chromatin. Further characterization of the shed cells using an antibody (G11), which is reactive with syncytiotrophoblast

Table 1. Antibodies used in this study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host species</th>
<th>Dilution</th>
<th>Specificity</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokeratin-7</td>
<td>Mouse</td>
<td>1:200</td>
<td>Epithelial cells including trophoblast</td>
<td>DAKO, MedBio, Christchurch, New Zealand</td>
</tr>
<tr>
<td>CD45</td>
<td>Mouse</td>
<td>1:200</td>
<td>Leukocytes</td>
<td>DAKO, MedBio, Christchurch, New Zealand</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Mouse</td>
<td>1:400</td>
<td>Non-epithelial cells</td>
<td>DAKO, MedBio, Christchurch, New Zealand</td>
</tr>
<tr>
<td>M30 cyto death</td>
<td>Mouse</td>
<td>1:25</td>
<td>Apoptotic trophoblast</td>
<td>Roche Diagnostics, Auckland, New Zealand</td>
</tr>
<tr>
<td>Activated caspase-3</td>
<td>Rabbit</td>
<td>1:200</td>
<td>Apoptotic trophoblast</td>
<td>Sigma, Australia</td>
</tr>
<tr>
<td>iN2</td>
<td>Rabbit</td>
<td>1:200</td>
<td>Negative control</td>
<td>This laboratory</td>
</tr>
<tr>
<td>G11</td>
<td>Mouse</td>
<td>1:100</td>
<td>Syncytiotrophoblast</td>
<td>This laboratory</td>
</tr>
<tr>
<td>BO1D11</td>
<td>Mouse</td>
<td>1:100</td>
<td>Extravillous trophoblast</td>
<td>This laboratory</td>
</tr>
<tr>
<td>CD71</td>
<td>Mouse</td>
<td>1:200</td>
<td>Proliferating cells</td>
<td>DAKO, MedBio, Christchurch, New Zealand</td>
</tr>
</tbody>
</table>
**In vitro trophoblast shedding**

Semi-quantitative analysis of shed cells stained with an antibody reactive with the M30 cytokeratin neoepitope revealed that 38% (SE ± 1.42) of the mononuclear trophoblasts and 25% (SE ± 1.63) of the syncytial knots shed were apoptotic after 24 h in culture. The percentage of both types of shed trophoblast that were apoptotic increased significantly (P < 0.0001) with time in culture (Figure 4). The number of syncytial knots stained with an activated caspase-3-reactive antibody was not significantly different from those staining with the M30 antibody (data not shown). Counting MTT-stained shed cells (Figures 1 and 4) revealed that after 24 h, ~59% (SE ± 1.31) of the shed mononuclear trophoblasts and 71% (SE ± 1.89) of the syncytial knots were strongly MTT positive, suggesting that they were viable, but this percentage decreased significantly (P < 0.0001) with time in culture (Figure 4). The number of apoptotic trophoblasts and viable trophoblasts did not add up to the total number of trophoblasts shed from the explants, and we estimated the numbers of non-viable, non-apoptotic (necrotic) trophoblasts using the equation below:

\[
\text{Necrotic trophoblasts} = \text{total shed trophoblasts} - (\text{viable trophoblasts} + \text{apoptotic trophoblasts})
\]

Approximately 5% of both the mononuclear trophoblast and the syncytial knots shed from the cultured explants were necrotic, and this percentage remained relatively unchanged throughout the 3-day study period (Figure 4).

**The effects on trophoblast shedding of inhibiting caspases**

To further investigate the role of apoptosis in trophoblast shedding, we quantified and compared trophoblasts shed from villous explants that had been incubated in the presence or absence of a broad-spectrum caspase inhibitor. This analysis demonstrated that the caspase inhibitor significantly (P < 0.0001) reduced the numbers of both mononuclear trophoblasts and syncytial knots shed at all time points (Figure 5) but that the shedding of mononuclear trophoblasts was more dramatically effected than the shedding of syncytial knots. The caspase inhibitor significantly reduced the numbers of shed viable (MTT positive) mononuclear trophoblasts, by ~4-, 5- and 6-fold at 24, 48 and 72 h, respectively (Figure 6). Whereas the inhibitor reduced the numbers of viable syncytial knots by only approximately 2-fold at all time points; but this reduction was significant (P < 0.002). Conversely, the caspase inhibitor caused a striking increase in the percentage of necrotic trophoblasts shed especially at the 72-h time point when 66% (SE ± 6.15) of the mononuclear trophoblasts and 52% (SE ± 5.99) of the syncytial knots shed were necrotic (Figure 6).

**Discussion**

Despite trophoblast deportation having been first described by Schmorl over 100 years ago, little is known about the processes that lead to trophoblast deportation or the consequences of deported trophoblasts on maternal physiology, and this is largely because deported trophoblasts are inaccessible in vivo. Trophoblast deportation involves

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**Figure 1.** Phase-contrast photomicrograph showing an example of the medium harvested from the chamber below a villous explant cultured for 24 h in a Net-well insert demonstrating shed syncytial knots (arrowheads), mononuclear cells (arrow) and red blood cells (dashed arrow). Bar = 20 μm.

**Figure 2.** Photomicrographs demonstrating the characterization of cells shed from placental explants in vitro. (A) Mononuclear trophoblasts and syncytial knots stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), (B) G11 antibody stains syncytial knots (red) but not mononuclear trophoblasts (arrow), (C) mononuclear trophoblast stained with a cytokeratin-7-reactive antibody (red) and (D) an isotype-matched CD45-reactive antibody stains leukocytes (red) but not a syncytial knot (arrow). The shed cells were counterstained with haematoxylin. Bar = 30 μm.

but not with cytotrophoblast, as well as the lack of expression of CD71 by these mononuclear trophoblasts suggested that the shed mononuclear trophoblasts were not derived from the syncytium (Figure 2B). The shed RBCs were not nucleated.

**Quantification of trophoblast shedding**

Semi-quantitative analysis revealed that, after 24 h, there were a mean of 3.6 mononuclear trophoblasts and 1.9 syncytial knots shed per milligram of explant. The numbers of both mononuclear trophoblasts and syncytial knots then decreased at 48 h (P < 0.0001) but increased again at 72 h (P < 0.0001) (Figure 3). Conversely, the number of shed CD45-positive cells and vimentin-positive cells decreased significantly (P < 0.0001) with increasing time in culture. However, there was no significant difference between the numbers of vimentin-positive and CD45-positive mononuclear cells shed from the explants at any time point (P > 0.1), suggesting that all of the non-trophoblast mononuclear cells were leukocytes (data not shown).
at least two separate physical processes. One of these processes is the transportation of trophoblasts from the uteroplacental site to distal sites in the maternal body via the maternal blood. However, preceding this step, trophoblasts are first shed from the placental surface. In this study, we have developed an *in vitro* model of trophoblast shedding, which allowed us to study the biological factors involved in the shedding of trophoblasts and the nature of the shed trophoblasts. In establishing this model, we carefully considered several issues.
First, we choose to use placentae of 12-week gestation. This was because there is evidence suggesting that the oxygen concentration around the human placenta increases near the end of the first trimester of pregnancy when the trophoblast plugs in the spiral arteries dissipate, allowing maternal blood to enter the intervillous space (Rodesch et al., 1992; Merce et al., 1996; Jaffe, 1998; Watson et al., 1998; Jauniaux et al., 2000). Furthermore, we have recently shown, by studying >3500 explants from placentae ranging from 8 to 12 weeks of gestation, that although oxygen concentration affects the behaviour of trophoblasts from placentae of 10-week gestation or less, there is little or no effect of oxygen concentration on explants from placentae of ≥11-week gestation (James et al., 2006). In addition, although placentae of 12-week gestation are less susceptible to oxidative damage than placentae of earlier gestation, they retain a full/continuous mononuclear trophoblast layer beneath the syncytiotrophoblast and thus should have significant regenerative capacity (Rodesch et al., 1992; Merce

Figure 5. The mean numbers of cytokeratin-positive trophoblasts shed per milligram of villous explant from explants cultured in the presence (white) or in the absence (grey) of 5 μM caspase inhibitor. The mean number of trophoblasts shed per milligram of placental tissue decreased significantly in the presence of the caspase inhibitor (t-test, *P < 0.00001). Data were obtained from triplicate explants at each time point from 12 separate placentae. Bars represent SE.

Figure 6. Percentage of viable (white), apoptotic (grey) and necrotic (striped) trophoblasts that were shed per milligram of placental tissue.
et al., 1996; Jaffe, 1998; Watson et al., 1998; Jauniaux et al., 2000). Therefore, it seemed likely to us that placentae of 12-week gestation would require culture in reduced oxygen levels, as would be required for earlier gestation placentae; yet, they have substantial regenerative capacity that might have been reduced in later gestation placentae.

Second, the tissues were carefully washed to remove contaminating maternal cells. However, this washing was not entirely effective as demonstrated by the continued shedding of CD45-positive cells from the explants. That these CD45-positive cells were likely to be of maternal origin is suggested by their decreased numbers with time in culture, but we cannot rule out the possibility that these CD45-positive cells were of fetal origin. Regardless of whether these were fetal or maternal leukocytes, their shedding from the explants means that caution must be taken to avoid confusing these leukocytes with shed mononuclear trophoblasts. In this study, we either used trophoblast-specific markers to avoid this confusion or depleted the leukocytes using magnetic beads before the final counting of shed cells.

Third, the Net-well inserts we used allowed the suspension of the explants in the medium in a manner modelling the suspension of floating villi in the maternal blood lakes and allowed us to move the explants into fresh wells without the need to touch the delicate explants, thereby reducing experimental artefacts. This ability to move the explants allowed us ready access to the shed trophoblasts that fell through the 400-μm mesh at the bottom of the inserts.

Fourth, by gently shaking the explants before moving them to fresh culture wells each day, we hoped to imitate the effects of the movement of maternal blood across the villous surface, which is likely to be one of the forces involved in trophoblast shedding and deportation. Although this may introduce variation between individual experiments, the shaking was gentle and as uniform as possible. In addition, the large number of experimental replicates used should have diminished the effects of inter-experiment variation.

We and others have previously shown that the syncytiotrophoblast undergoes significant artefactual degeneration during the first 24 h of villous explant culture and that it is subsequently regenerated (Palmer et al., 1997; James et al., 2005). Confirming the artefactual degeneration of the syncytiotrophoblast during the first 24 h of explant culture, we demonstrated that there was significantly more trophoblast shed at the 24-h time point than at later time points in our model.

However, surprisingly, we found that most of these artefactually shed trophoblasts were still able to metabolize MTT, suggesting that they were viable. We had anticipated that these cells would be shed because of exposure to excess (atmospheric) oxygen levels or the effects of the physical trauma of the TOP and that, consequently, they would be necrotic. Although the ability of a cell to metabolize MTT is often used as a marker of cell viability, it is actually a marker of mitochondrial function, and it may be that many of these artefactually shed trophoblasts were undergoing a death process but that this process was not far enough advanced to have caused either mitochondrial dysfunction or caspase activation. Future studies using other markers of cell viability/death will be required to clarify this issue.

These results suggest that the process by which syncytial knots are extruded from the placental surface is not absolutely dependent upon the ‘death’ of either the syncytiotrophoblast or the mononuclear trophoblasts. We believe that it is crucial to bear this artefactual shedding in mind when looking at our results (as well as the results of others) and believe that the results of the trophoblast shedding that occurred at 48 and 72 h in our model are more likely to be a truer representation of shedding as it occurs in vivo. We have also shown in other work (James et al., 2005), as well as by examining the explants used in this study, following MTT staining (data not shown), that the viability of most cells in villous explants is good between 24 and 72 h of culture but that the viability of most of the cells in villous explants is significantly reduced after 96 h in culture, and consequently, for the remainder of our discussion, we will consider only those trophoblasts shed at 48 and 72 h. Both mononuclear cytotrophoblasts and multinucleated trophoblasts (syncytial knots) were shed from the explants, and the trophoblasts that were shed in our model closely resemble those that have previously been reported by others to be shed in vivo (Chua et al., 1991; Hawes et al., 1994; Johansen et al., 1999). That the mononuclear trophoblasts were not fragments of the syncytiotrophoblast was confirmed by their failure to stain with the G11 antibody, which reacts with syncytiotrophoblast but not with cytotrophoblasts, as well as by the absence of CD71 expression by the shed mononuclear trophoblasts (data not shown). It is most likely that these mononuclear trophoblasts were villous cytotrophoblasts because there were likely to be very few extravillous cytotrophoblasts associated with the explants we used. It is not clear to us why villous cytotrophoblasts should be shed from the placenta because they are usually overlain by the syncytiotrophoblast, but it is possible to speculate that they are shed accidentally after being exposed at syncytiotrophoblast discontinuities during the process of syncytiotrophoblast shedding and subsequent regeneration (Nelson, 1996). Alternatively, it is possible that cytrophoblasts are shed ‘deliberately’ as part of the mechanism for extruding syncytial knots from the placental surface. Further work will be required to establish why, both in vitro and in vivo, cytotrophoblasts are shed from the placenta.

Based on published mean placental weights (Kaufmann, 2000) and the mean numbers of trophoblasts shed from the explants in our study, we estimate that 10^7 mononuclear trophoblasts and 4.7 × 10^6 syncytial knots would be shed daily from a placenta of 12-week gestation. The numbers of trophoblasts shed daily by an average placenta at 9 months of gestation would rise to 1.8 × 10^8 mononuclear trophoblasts and 8.5 × 10^6 syncytial knots. Our estimates are greater than the widely quoted estimate of 150 000 trophoblasts shed daily during normal pregnancy, but we have been unable to determine how Chua et al. (1991) derived the quoted estimates, and they have acknowledged that 150 000 may be a substantial underestimate of daily trophoblast shedding. Our estimates are based on the assumptions that (i) our model accurately reflects the levels of trophoblast shedding occurring in vivo (these estimates were based on the mean numbers of trophoblast shed in our model at 48 and 72 h) and (ii) that the rate of shedding is directly related to placental weight. This latter assumption is somewhat questionable because the internal structures of the placenta, in particular the relative amount of mononuclear trophoblast, change with increasing gestation age. It would be of significant value to repeat experiments such as those we describe here using mid-trimester, as well as term placenta to address this issue directly.

The manner in which we have presented our data may lead the casual observer to believe that shedding of mononuclear trophoblasts is quantitatively more important than that of syncytial knots. This is because we have reported the numbers of each of these cell types. It must be borne in mind that each syncytial knot contains up to several hundred nuclei and vastly more cytoplasm than mononuclear trophoblasts, and had we been able to quantify the numbers of nuclei, it would be quite clear that the total amount of cellular material shed as syncytial knots greatly exceeds the amount of material shed as mononuclear trophoblasts. However, because of the large physical size of the shed syncytial knots, it was impossible to accurately quantify the numbers of shed nuclei in these structures.

Microscopic examination demonstrated that the nuclei of the shed trophoblasts were densely packed, suggesting that they may have been apoptotic. We confirmed that these cells were undergoing an apoptotic process by staining for both the M30 cytokeratin neoepitope and the activated caspase-3. This suggested that, as has been hypothesized by others, apoptosis plays a major role in the shedding of these cells.
from the placenta (Nelson, 1996; Huppertz et al., 1998). To further examine the role of apoptosis in the process of trophoblast shedding, we studied the effects of simultaneously inhibiting caspases 3, 6, 7, 8 and 10. This resulted in substantial reductions in the number of trophoblasts shed from explants, confirming that most shed trophoblasts were apoptotic. The mechanism that physically drives the shedding of trophoblasts from the villous surface is at present unknown. That inhibiting caspases caused a significant reduction in the total number of trophoblasts shed indicates that the apoptosis pathway may be important in driving the shedding process. However, because shedding was not abolished by the caspase inhibitor, it seems that other pathways must also be involved in the process of trophoblast shedding, and there may be redundancy between these alternative pathways.

Although several mechanisms have been proposed to explain why trophoblasts are shed into the maternal blood, our results and those of others (Ku et al., 1997; Mayhew et al., 1999; Kadyrov et al., 2001) suggest that the most likely explanation for shedding of trophoblasts is simply that shedding is the final stage in the normal cellular ageing and turnover of the trophoblast layers and is analogous to shedding of other epithelia as has been suggested previously (Huppertz and Kingdom, 2004; Zbar et al., 2004).

Although several other workers have suggested that shed trophoblasts are likely to be apoptotic (Huppertz et al., 2003; Huppertz and Kingdom, 2004), we believe our study provides the first direct experimental documentation that shed trophoblast is predominantly apoptotic. Two further points worthy of discussion arise from these experiments. First, the shedding of mononuclear trophoblasts was reduced to a much greater extent than that of syncytiotrophoblast. This could be explained if the apoptotic pathway had already been partially initiated in the syncytiotrophoblast such that there was a smaller range of caspase-dependent steps that remained to be inhibited in the syncytiotrophoblast than in the cytotrophoblasts and supports the suggestion that syncytiotrophoblasts, before their extrusion from the placenta, are proapoptotic (Mayhew et al., 1999). Second, although the caspase inhibitor caused notable and significant reductions in the total numbers of both syncytiotrophoblast and cytotrophoblasts shed, a more striking change occurred in the nature of the dead trophoblasts that changed from being predominately apoptotic to being predominately necrotic. This suggests to us that the trophoblasts were committed to die and that, when the apoptotic pathway was unavailable, the ageing trophoblasts had to die by an alternative mechanism such as necrosis or by a combination of death mechanisms, which may be analogous to aponecrotic death as proposed by Huppertz et al. (2003). Alternatively, caspase-independent programmed cell death may also contribute to the shedding of trophoblast when the caspase pathways are inhibited. Regardless of the exact mechanism of death, this finding strongly suggests that trophoblasts have a programmed life span.

Although most of theshed trophoblasts were apoptotic, we were interested to know more about the condition of the non-apoptotic cells. Consequently, following the depletion of contaminating leukocytes, we determined whether any of the shed trophoblasts were viable, by staining with MTT, and found that 6% of both the mononuclear trophoblasts and the syncytiotrophoblasts shed from the cultured explants were viable after 72 h of culture (Figure 4). Some of these viable syncytiotrophoblasts may be true syncytiotrophoblasts which have detached from the placental surface as suggested by Boyd and Hamilton (1970), but because the placentas we studied were of 12-week gestation, this seems unlikely. There is growing evidence that fetal cells remain viable in maternal organs and/or the maternal circulation for prolonged periods of time after pregnancy (Bianchi et al., 1996; O’Donoghue et al., 2004). This phenomenon is called chronic microchimerism. While other cell types, such as fetal mesenchymal stem cells, are known to be involved in fetomaternal microchimerism, our results suggest that trophoblasts could also contribute to the chronic microchimerism induced by pregnancy. Given the possibility that some of these viable trophoblasts could be true syncytiotrophoblasts and the possibility that they could contribute to chronic microchimerism, it would be interesting, in future studies, to determine exactly how long trophoblasts remain viable, in vitro, after they have been shed.

The cumulative numbers of viable and apoptotic trophoblasts shed from the explants was less than the total number of shed trophoblasts, suggesting that a small percentage (~5%) of shed trophoblasts were neither viable nor apoptotic, and we suggest that these trophoblasts died by a caspase-independent mechanism and may have been necrotic. Unfortunately, there are no objective markers that can confirm that these trophoblasts were necrotic, but this finding is potentially very significant to the outcome of pregnancy because evidence from other systems suggests that apoptotic cells are phagocytosed without stimulating an inflammatory immune response or may lead to activation of immune responses to antigens from the apoptotic cells (Fadok and Chimini, 2001). In contrast, phagocytosis of necrotic cells can lead to development of inflammatory immune responses. Thus, phagocytosis of shed apoptotic trophoblasts by maternal immune cells may produce a suppressive type of immune response, which would be beneficial to the maintenance of the pregnancy. Indeed, we have recently shown that trophoblasts shed from our model produce an anti-inflammatory, immunosuppressive type of immune response (Abumaree et al., 2006). We have also recently shown that endothelial cells can phagocytose dead trophoblasts (Chen et al., 2006). Although phagocytosis of apoptotic trophoblasts had no effect on the endothelial cells, phagocytosis of necrotic trophoblasts led to endothelial cell activation (Chen et al., 2006). Given that increased trophoblast shedding is thought to occur in pre-eclampsia and that both endothelial activation and aberrant inflammatory responses are hallmarks of pre-eclampsia, increased shedding of necrotic trophoblasts may be important to the pathogenesis of this disease.

In summary, we report a novel placental villous explant model that allows the study of trophoblast shedding from the human placenta. This model is likely to be useful for studying the causes and consequences of trophoblast deportation.

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References

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