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The rate of trophoblast shedding/deportation alters with increasing gestational age: An *in vitro* model to quantify trophoblast deportation

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ABSTRACT

Objective: To quantify trophoblast deportation after developing an *in vitro* model of trophoblast deportation by culturing placental explants in Net-well inserts which allow passage of cells shed from the explants to the lower chamber from where we can harvest, characterize and quantify the shed trophoblasts. **Methods:** Our original model employed explants from placentae of 12 weeks gestation. Using that model, we estimated about 150 000 trophoblasts would shed daily from a placenta of 12-week gestation. In this study, we determined whether trophoblast shedding is consistent throughout pregnancy. Placental explants from second-trimester and term placentae were cultured in Net-well inserts for 72 hours. Shed cells were characterized by immunocytochemistry and quantified. Published mean placental weights were then used to extrapolate an estimate of the rates of trophoblast shedding *in vivo*. **Results:** Syncytial nuclear aggregates and mononuclear trophoblasts were shed from the second-trimester and term placentae. We also noted the shedding of cytokeratin-positive anucleate "ghosts" which we presume to be derived from syncytial nuclear aggregates. Relative to the numbers of trophoblasts shed from first trimester placentae, the numbers shed from second trimester placentae declined but the number of trophoblasts shed increased again from third trimester placentae. **Conclusion:** We have shown that the rate of trophoblast shedding is inconsistent throughout gestation confirming the results of others who found variable levels of deported trophoblasts in maternal blood.

1. Introduction

Trophoblast deportation, the process whereby trophoblasts that are shed from the placenta are transported to distal sites via the maternal blood, was first documented more than a century ago by Schmorl [1]. Subsequently, it has been shown that trophoblast deportation is a physiological feature of normal pregnancy that may be exacerbated in preeclampsia, eclampsia and other diseases of pregnancy[2-6]. It is thought that the majority of 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 occurs in the gut [7]. Several reports have been suggested that programmed cell death (apoptosis or

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a variant of that process) may initiate degeneration in the syncytiotrophoblast as this layer naturally ages leading to shedding of multinucleated fragments called syncytial nuclear aggregates[8-14].

Recently, we have developed an *in vitro* model of trophoblast deportation/shedding, based on culture of villous explants from first trimester placentae [15]. This model allowed us to quantify the number of trophoblast shed per mg of villous explant and also to determine the role of apoptosis in trophoblast shedding/deportation [15]. From this *in vitro* data we were able to extrapolate the rates of trophoblast shedding/deportation that might occur *in vivo* during different stages of pregnancy [15]. However, our extrapolations were based on a number of assumptions. In this study, we adapted our *in vitro* model to quantify trophoblast deportation using villous explants from second-trimester, and term placentae in order to examine, then validate some of our earlier assumptions.

2. Materials and methods

2.1. Ethics of experimentation

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

2.2. Placentae

Mid trimester and term placentae were obtained following elective surgical termination of pregnancy (TOP) and normal vaginal delivery, respectively. The gestational age and fetal viability of all pregnancies were confirmed by early ultrasound examination before 20 weeks gestation.

2.3. Harvesting trophoblasts shed from placental explants in vitro

Placental tissue from second-trimester placentae (14, 15, and 17 weeks of gestation) and term placentae was washed with ice-cold PBS, dissected, rewashed with ice-cold PBS and triplicate explants (individual explants weight is approximately 40 mg) were transferred into Net-well™ inserts (400 μm mesh) in 12 well culture plates with 3 mL of DMEM/F12 Medium containing 10% Fetal Bovine Serum (FBS), 5 ng/mL epidermal growth factor, 5 μg/mL insulin, 10 μg/mL transferin, 100 μg/ml L-Glutamate, 20 nM sodium selenite, 400 U/l hCG, 100 μg/mL streptomycin, and 100 U/L penicillin. The explants were cultured at 37 ℃ in a humidified atmosphere containing 5% CO_2 and 95% air for 72 hours. Every 24 hours, the culture plates were gently shaken and the Net-well™ inserts containing the explants were transferred to fresh culture wells containing fresh medium. Shed cells passed from the Net-well™ insert under gravity and were harvested by aspiration and transferred to a 1.5 mL microfuge tube then centrifuged at 480×g for eight minutes at 20 ℃. Shed cells were resuspended in 210 μL of PBS then 30 μL aliquots were air-dried onto microscope slides and fixed with cold acetone (-20 ℃) for 10 minutes and then left to air-dry for 1 hours. Slides with acetone-fixed air-dried trophoblasts were stored at -20 ℃ until characterization by immunocytochemical staining.

2.4. Immunocytochemistry staining

Slides with the air-dried cells were thawed for 5 minutes at room temperature and the cells were encircled with a Dako pen (DAKO, MedBio, New Zealand). Non-specific binding was blocked by incubation with 10% normal goat serum in PBS-Tween for 10 minutes at room temperature. After three washes with PBS-Tween, the slides were incubated with primary antibodies and with irrelevant, class-matched, control antibodies diluted in 10% normal goat serum in PBS-Tween as shown in Table 1 for 1 hour at room temperature. The antibodies used in this study targeted the cytokeratin 7, CD45, and vimentin antigens. These primary antibodies were purchased from DAKO, MedBio, Christchurch, New Zealand. The slides were then washed three times with PBS-Tween and the activity of endogenous peroxidase was quenched by the addition of 3% H_2O_2 in methanol for 5 minutes at room temperature. The slides were then washed three times with PBS-Tween. A Zymed Histostain-Plus kit (Zymed, San Francisco, CA, USA) containing biotinylated secondary antibody and enzyme conjugate was used according to the manufacturer's instructions. Amino ethyl carbolyl stain (MedBio, Christchurch, New Zealand) was used according to the manufacturer's instructions. Slides were then washed with de-ionized water, immersed in haematoxylin nuclear stain (Surgipath; Sigma, Sydney, Australia,) for 1 minute then washed with tap water and coverslips were mounted with Aquamount. The numbers of cells shed from the explants were quantified by counting the number of stained cells in ten random high power fields (40 ×) on the immunocytochemistry slides. Then the number was expressed as the mean number of shed cells per mg placental tissue in the explants following the equation:

$$n = \frac{\text{Mean number of shed trophoblasts per slide} \times 7 \text{ (number of slides)}}{120\text{mg (total weight of 3 explanted villous tissues)}}$$

Table 1
Antibodies used in this study.

Antibody	Host Species	Dilution	Specificity	Manufacturer
Cytokeratin-7	Mouse	1:200	Villous cytotrophoblasts, syncytiotrophoblasts and extravillous trophoblasts	DAKO, MedBio, Christchurch, New Zealand
CD45	Mouse	1:200	Leucocytes	DAKO, MedBio, Christchurch, New Zealand
Vimentin	Mouse	1:400	Fibroblasts	DAKO, MedBio, Christchurch, New Zealand

2.5. Statistical analysis

Data were analyzed using the student's t test or ANOVA as appropriate. These analyses were performed using Microsoft EXCEL (Office 97 software). Results were considered to be statistically significant if $P<0.05$.

3. Results

3.1. Characterization of shed cells

Analysis of the immunocytochemically stained cells shed from the explants every 24 hours showed that leucocytes, red blood cells (RBCs) and two distinct types of trophoblasts, mononuclear trophoblasts and multinucleated syncytiotrophoblast fragments that we referred to as syncytial nuclear aggregates were shed (Figure 1A and 1B). Neither the mononuclear trophoblasts nor the syncytial nuclear aggregates stained with class-matched control antibody reactive with CD45 (Figure G). In addition to these trophoblasts, we noted a number of apparently cytokeratin-containing anucleated structures which, given their size, might have been "ghosts" of syncytial nuclear aggregates, but because of our uncertainty as to their nature we did not quantify their shedding (Figures 1C, 1D, 1E). There were additional "ghosts" that did not contain cytokeratin.

It was interesting to note that syncytial nuclear aggregates appeared to have two quite distinct cytokeratin staining patterns. In the first, the cytokeratin staining was distributed relatively evenly around the knots and in the second, the cytokeratin appeared to be heavily concentrated at one poll of the knot (Figure 1B, 1C). The putative syncytial nuclear aggregate ghosts also had this dichotomous cytokeratin staining pattern.

3.2. Quantification of trophoblast shedding

3.2.1. Second trimester placentae

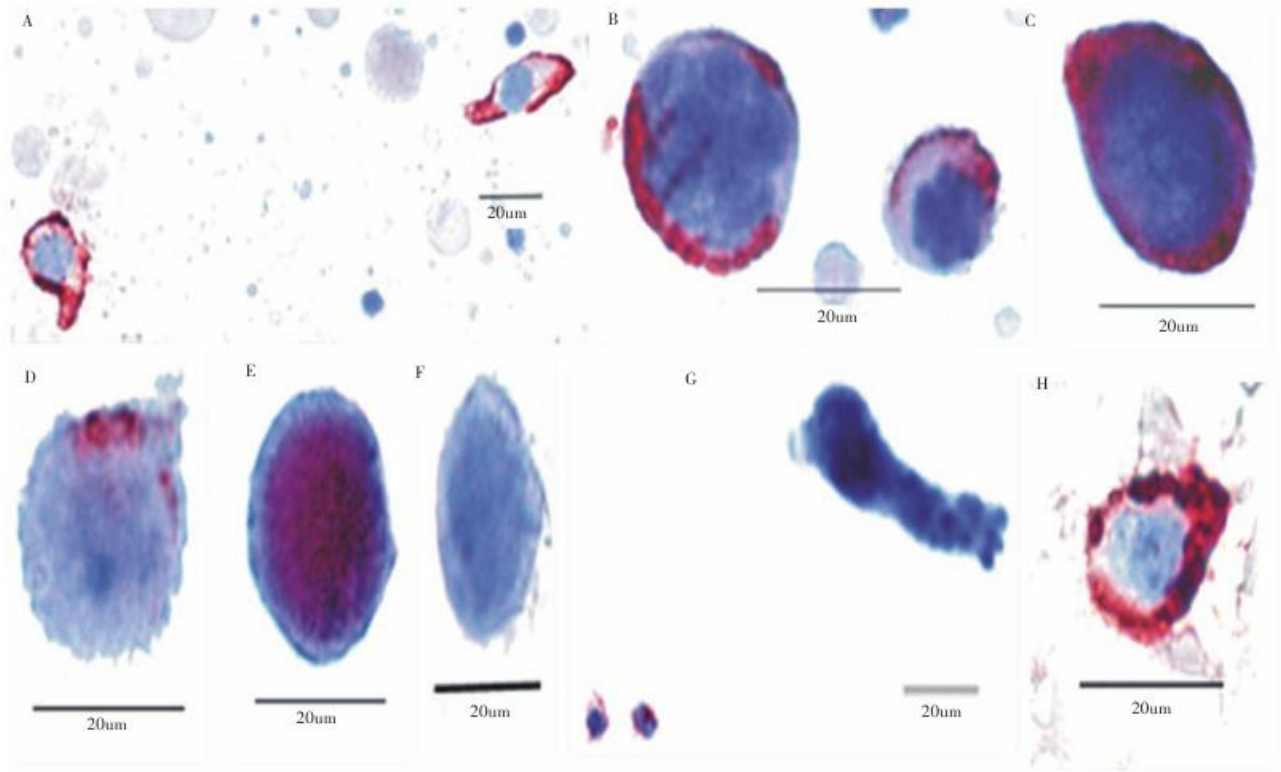

Figure 1. Photomicrographs demonstrating the characterization of cells shed from second-trimester and term placental explants *in vitro*. Mononuclear trophoblasts stained with a cytokeratin 7-reactive antibody (A). Syncytial nuclear aggregates stained with a cytokeratin 7-reactive antibody demonstrating localised (B) or diffuse staining (C). Ghosts of syncytial nuclear aggregates stained with the cytokeratin-7- reactive antibody also demonstrated both localised (D) and diffuse staining (E). Ghosts (F) and syncytial nuclear aggregates (G) did not stain with a class-matched antibody reactive with CD45 but contaminating leucocytes did (g arrows). Binucleated cytokeratin-7 positive cells were also rarely shed from the explants (H).

Semi-quantitative analysis revealed that after 24 hours there were a mean of 1.04 mononuclear trophoblasts and 0.46 syncytial nuclear aggregates shed per mg of explant from second trimester placentae. The numbers of both mononuclear trophoblasts and syncytial nuclear aggregates then decreased at 48 hours ($P<0.01$) but increased again at 72 hours ($P<0.05$) (Figure 2).

3.2.2. Term placentae

Semi-quantitative analysis revealed that after 24 hours there were a mean of 0.33 mononuclear trophoblasts and 0.10 syncytial nuclear aggregates shed per mg of explant from term placentae. The numbers of both mononuclear trophoblasts and syncytial nuclear aggregates then decreased at 48 hours ($P<0.01$) but increased again at 72 hours ($P<0.05$) (Figure 3).

3.2.3. The pattern of trophoblast shedding through pregnancy

Based on our previous data[15], and the data presented above, we were able to estimate the numbers of mononuclear

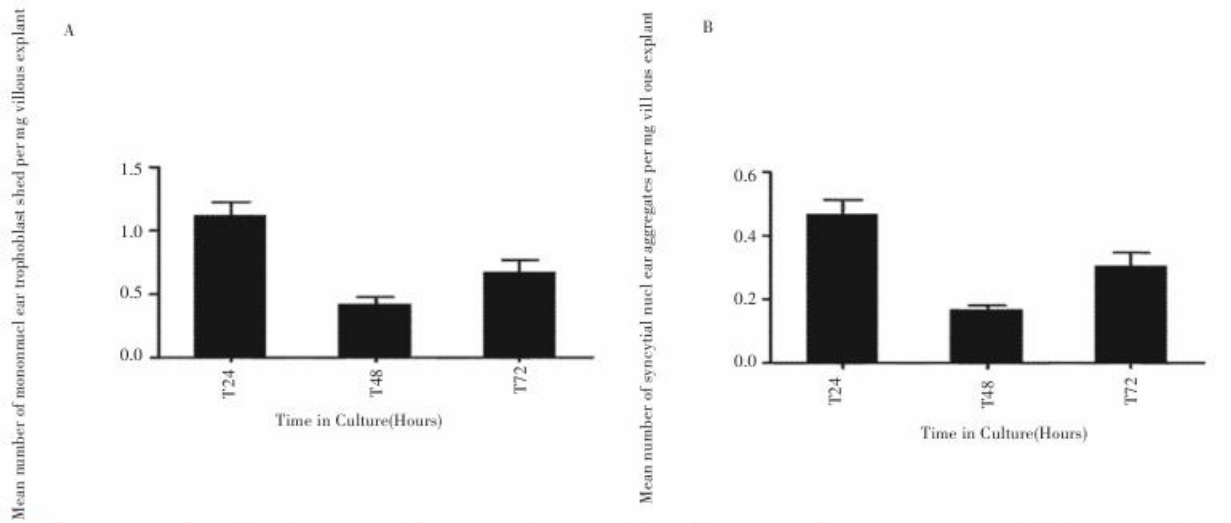

Figure 2. The mean numbers of cytokeratin-positive mononuclear trophoblasts (A) or syncytial nuclear aggregates (B) shed per mg villous explant from second-trimester. The number of shed mononuclear trophoblasts and syncytial nuclear aggregates changed significantly with time ($P<0.001$). Each experiment was conducted using triplicate villous explants from 16 separate placentae. Bars represent standard errors.

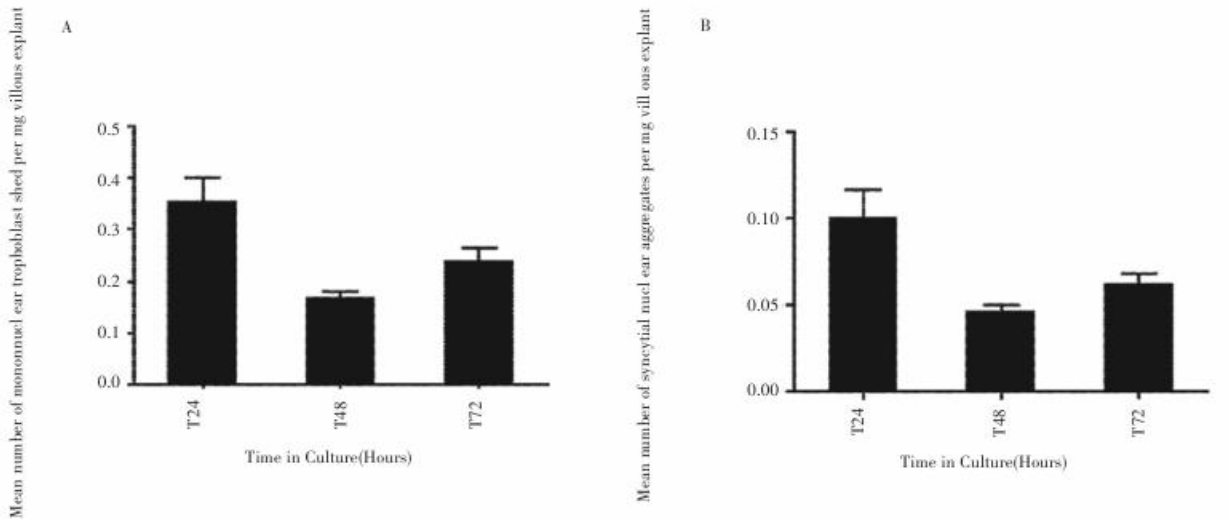

Figure 3. The mean numbers of cytokeratin-positive mononuclear trophoblasts (A) or syncytial nuclear aggregates (B) shed per mg villous explant from term placentae. The number of shed mononuclear trophoblasts and syncytial nuclear aggregates changed significantly with time ($P<0.001$). Each experiment was conducted using triplicate villous explants from 16 separate placentae. Bars represent standard errors.

trophoblasts and syncytial nuclear aggregates that might be shed into the maternal blood at various stages of pregnancy (Figure 4, 5). We have used only the mean numbers of trophoblasts shed at 48 and 72 hours to estimate trophoblast shedding *in vivo*.

4. Discussion

We have used our *in vitro* model of trophoblast shedding/deportation which allows us to study the biological factors involved in trophoblast deportation [15]. In our previous work with this model, we used villous explants from placentae of 12 weeks gestation only [15-16]. We specifically chose placentae of that gestational age, because those placentae contain a complete bilayer of trophoblasts, and therefore we reasoned it would have significant ability to regenerate any shed syncytiotrophoblast (syncytial nuclear aggregates). At twelve weeks of gestation, it was also likely that the placentae had been exposed to maternal blood after the dissipation of the trophoblast plugs, which occlude the spiral arteries prior to that gestation. Using that model, we were able to show that both syncytial nuclear aggregates and a surprisingly large number of mononuclear cytotrophoblasts were shed from the explants and these shed cells closely resembled those that had previously been reported by others to be shed in vivo [4, 6, 17]. We were also able to calculate the numbers of these two cell types shed from placentae of 12 weeks gestation [16, 18]. Based on the published weights of placentae, we extrapolated our data to estimate the numbers of trophoblasts shed/deported at different gestational ages. However, in performing those calculations we raised the

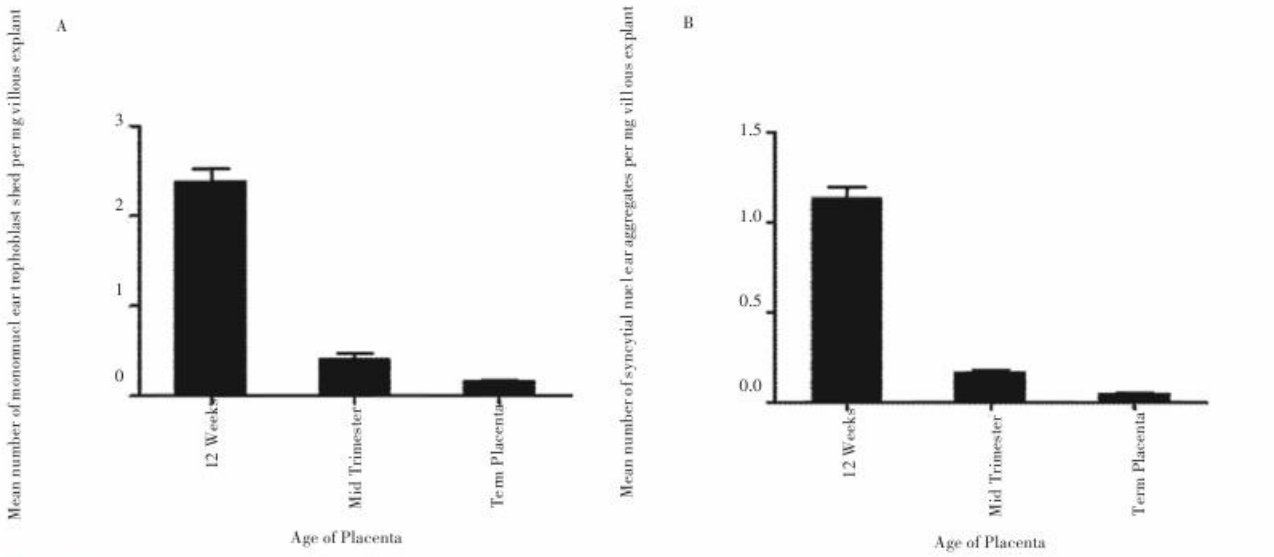

Figure 4. The mean numbers of cytokeratin-positive mononuclear trophoblasts (A) or syncytial nuclear aggregates (B) shed per mg villous explant from first trimester, second-trimester and term placentae. Bars represent standard errors.

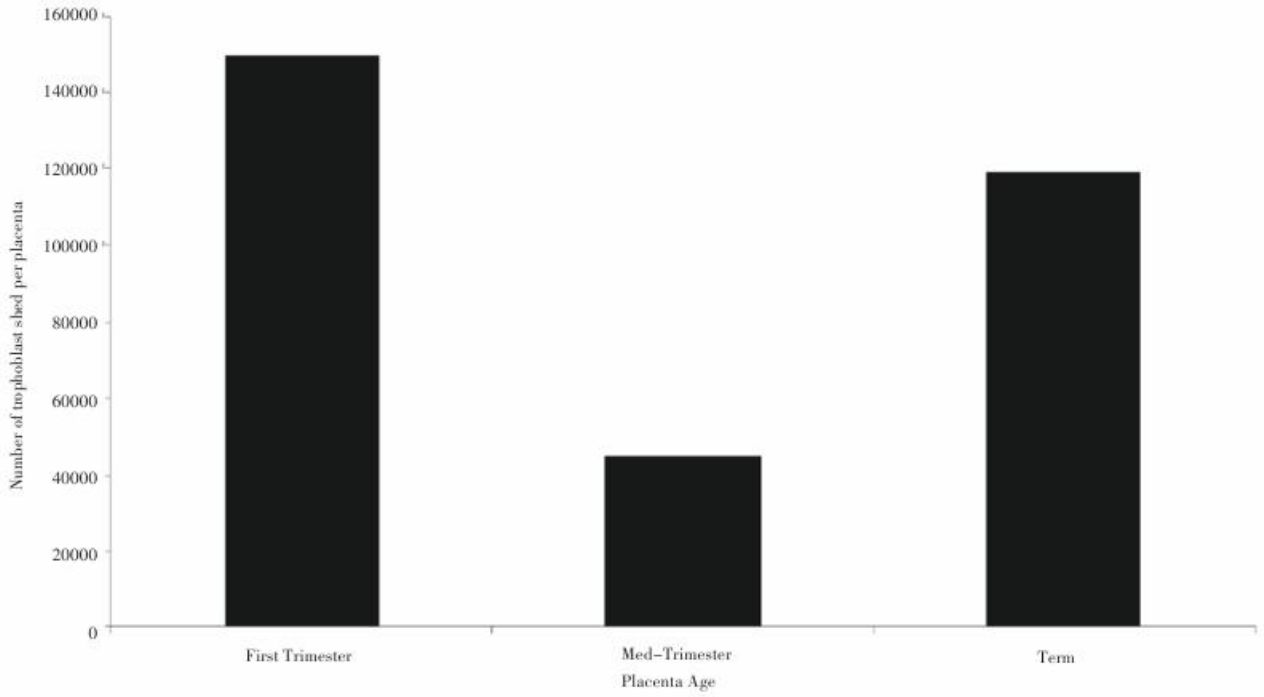

Figure 5. The estimated mean numbers of trophoblasts (combined mononuclear trophoblasts and syncytial nuclear aggregates) shed into the maternal blood based on our in vitro shedding rates including our previously published data and published mean placental weights.

caveat that our estimates were based on the assumption that the rate of trophoblast shedding is directly related to placental weight. Clearly placental structure changes significantly throughout gestation, but particularly the layer of cytotrophoblasts underlying the syncytiotrophoblast becomes less apparent and the relative contribution of this layer to total placental weight therefore decreases. This study was conducted in order to eliminate our flawed assumption, and provide direct experimental estimates of the rate of trophoblast shedding from later gestation placentae.

Given the structural changes in the placenta, we were not surprised to find that the rate of trophoblast shedding/mg of tissue declined with increased gestational age, but we were surprised by the pattern of change which suggests that there would be less shedding in the early part of the second trimester than in the later part of the first trimester. Furthermore, even at term, the numbers of trophoblasts shed was slightly less than from placentae of 12 weeks gestation. However, several groups have shown that the numbers of trophoblasts in maternal blood does not correlate

with gestational age, and potentially our results showing an unexpected pattern of trophoblast shedding could explain those earlier *in vivo* observations [6, 19].

In this study, we have presented the numbers of trophoblasts shed from the explants at each of the three time points during culture that we examined (24, 48 and 72 hours) for completeness. However, since it is now widely accepted that there is a substantial artifactual loss of the syncytiotrophoblast from placental explants during the first 24 hours of culture [14, 16, 19], we have used only the mean numbers of trophoblasts shed at 48 and 72 hours to estimate trophoblast shedding *in vivo*. The total numbers of shed trophoblast at term of approximately 120 000/day calculated by us is close to the widely quoted estimate of 150 000/day [20], but much lower than the estimate by Chua *et al* of up to 107 cells/hour.

We were surprised in our earlier study by the numbers of mononuclear trophoblasts that were shed from explants, but this study confirms that, on a numeric basis, there are more mononuclear trophoblasts than syncytial nuclear aggregates shed throughout gestation. However, it must be remembered that each syncytial nuclear aggregates contains substantially more nuclear and cytoplasmic material than the individual mononuclear cells. Why these mononuclear trophoblasts are shed remains unclear to us and this matter requires further investigation especially since, even at term when the cytotrophoblast layer is substantially less prominent than in early or mid-pregnancy, the number of shed mononuclear trophoblasts was numerically greater than the number of shed syncytial nuclear aggregates. While it is possible that the shedding of mononuclear trophoblasts we observed is in part an artifact induced by our experimental conditions, it is quite clear from the literature that mononuclear trophoblasts are shed into the maternal blood in vivo as several authors have reported isolating these cells from the maternal blood [4, 6-7, 17] and that is also our own (unpublished) experience. Furthermore, Chua et al reported that in the sample of blood with the highest numbers of trophoblasts that they obtained (51.5 cells/ml), the high numbers were due to mononuclear trophoblasts [6] suggesting that significant numbers of mononuclear trophoblasts are shed *in vivo* as well as *in vitro*.

In this study we also observed, infrequent cytokeratin positive cells which appeared to contain two nuclei. These cells were of similar size, and their nuclei appeared similar to those of the shed mononuclear trophoblasts. These bi-nucleated cells may be similar to the clusters of mononuclear trophoblasts found in the blood of pregnant women by Johansen *et al* but their rarity suggests they do not contribute significantly to the burden of shed trophoblasts [4, 6, 12].

When harvesting the cells shed from our explants, particularly from term placental explants, we noted large cytokeratin-containing cell-like structures that did not contain nuclear material. Due to their size, we believe these structures may be "ghosts" of syncytial nuclear aggregates, that is, membranes from syncytial nuclear aggregates which have lost their intracellular contents. We did not attempt to count these trophoblast ghosts, because we were concerned that they may take up the cytokeratin antibody non-specifically, and therefore be irrelevant (although we observed both cytokeratin positive and negative ghosts) and also because we are uncertain as to whether they represent true trophoblast shedding or an experimental artifact. However, similar structures were reported to be present in the blood of pregnant women by Covone *et al* and Johansen *et al*. The shed/deported anucleate trophoblasts reported by Covone et al in vivo were smaller than the majority of the ones we observed in vitro, but that may reflect the gentler conditions that the ghosts encountered in our in vitro system which could allow them to survive more intact than they would in the blood. As in our study, Covone et al also observed that the anucleated trophoblast ghosts were more abundant at term than earlier gestations. The similarity between the ghosts we have seen *in vitro* and those reported to exist *in vivo* suggests that attention should be paid to these structures in future studies. It was also striking that the two patterns of cytokeratin staining (diffuse and localised) we saw in syncytial nuclear aggregates was repeated in the ghosts further suggesting to us that these anucleate structures are indeed syncytial nuclear aggregates ghosts. Such ghosts may be one of the sources of fetal cell-free DNA in the maternal blood.

It is intriguing to speculate that the two distinctive cytokeratin staining patterns we observed in syncytial nuclear aggregates might represent different stages of degeneration of the syncytial nuclear aggregates. Schette *et al* have shown that after cleavage of cytokeratin 18 by execution caspases, the intermediate filament network collapses and the cellular keratins aggregate in apoptotic bodies [4, 21]. The localised "polar" cytokeratin staining that we have observed in some syncytial nuclear aggregates may represent the final collapse of the cytoskeleton whereas, syncytial nuclear aggregates with more diffuse staining are likely to represent earlier stages in the degeneration process. That syncytial nuclear aggregates at these differing stages of degeneration exist seems to confirm the earlier suggestion that syncytial nuclear aggregates are shed in a pro-apoptotic state rather than at the end stage of this death process [21-22].

In summary, we report that the in vitro rate of trophoblast shedding/mg of placental tissue decreases in the second trimester, then increases again at term. If these findings are reflective of the rates of shedding in vivo, this would explain why others have reported that the numbers of trophoblasts

in the blood of pregnant women do not correlate with gestational age.

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